Vol. IX, No. 4 December 1915

THE BIOCHEMICAL JOURNAL

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CAMBRIDGE UNIVERSITY PRESS
C. F. CLAY, Manager
LONDON: Fetter Lane, E.C.
EDINBURGH: 100, Princes Street

also

H. K. LEWIS & CO., Ltd., 136, Gower Street, London, W.C.
WILLIAM WESLEY & SON, 28, Essex Street, London, W.C.
CHICAGO: The University of Chicago Press
BOMBAY, CALCUTTA AND MADRAS: Macmillan and Co., Ltd.
TORONTO: J. M. Dent & Sons, Ltd.
TOKYO: The Maruzen-Kabushiki-Kaisha

1915

Price Seven Shillings net.

The *Biochemical Journal* is conducted by the Biochemical Society and is published by the Cambridge University Press.

This Society has been instituted for the purpose of facilitating intercourse between those biologists and chemists who are interested in the investigation of problems common to both, such as the chemical problems connected with Agriculture, Brewing, Animal and Vegetable Physiology and Pathology, &c. Persons interested in Biochemistry are eligible for election.

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The Journal is issued quarterly and the date at which each paper is received by the editors is printed at the beginning of the paper.

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XLI. THE INFLUENCE OF FAT AND CARBOHYDRATE ON THE EXCRETION OF ENDOGENOUS PURINES IN THE URINE OF DOG AND MAN.

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(*Received September 17th, 1915.*)

PART I.

In the year 1897 Camerer [1897] showed for the first time that there are two sources of origin for the uric acid in the urine of mammals, viz. (1) the nucleins of the tissues which are destroyed in the course of intracellular metabolism, and (2) the nucleins contained in food. In 1900 Burian and Schur [1900] distinguished these two sources of urinary purines as endogenous and exogenous. As regards the endogenous moiety of the urinary purines, Burian and Schur came to the conclusion that under normal conditions, when the calorie value of the purine-free diet is sufficient, the output of purine is quite independent of the total amount of nitrogen excreted and further that it remains a constant for each individual, although the range of variation in different individuals may be fairly wide. They believed however that the endogenous purine output might be changed by abnormal conditions. About the same time but independently Siven [1901] came to practically a similar conclusion. It is worthy of note that even in 1888 when the genetic relation between the purine compounds and uric acid was not fully appreciated Maréš [1888] had drawn attention to the constancy of the individual excretion of uric acid and to the fact that the amount excreted varied in different individuals. This constancy in the output of endogenous purines was confirmed by Schreiber and Waldvogel [1899], Rockwood [1904], Macleod

and Haskins [1906], Walker Hall [1904], Kaufmann and Mohr [1902] and Pfeil [1903].

Folin [1905] from his experiments, in which he used two diets, both purine-free, but one rich in protein and the other poor, came to the conclusion that the uric acid output is to a certain extent dependent on the protein content of the diet; with a reduction in the nitrogen excretion there is a corresponding reduction in the output of uric acid. Smetanka [1911] found an increased output of uric acid after the ingestion of protein and of carbohydrate, and Rosenfeld and Orgler [1896] noted that the output of uric acid was much increased when fat or carbohydrate was added to a diet consisting wholly of meat.

Cathcart [1907] observed that during fasting there was at first a fall in the excretion of uric acid but after a few days it gradually increased until it equalled the original amount. In another series of experiments this worker [1909] found that when a subject was put on a purine-free, carbohydrate-rich diet the uric acid excretion was remarkably high whereas when the diet was changed to one in which fat predominated the excretion of uric acid fell to a very low figure. Recently Graham and Poulton [1913] published a paper in which they described the influence of fats and carbohydrates on the endogenous purine output. They also observed that a fat-rich diet was associated with a low and a carbohydrate-rich diet with a high purine output. They ascribed the high output on the carbohydrate diet to a synthesis of purine from the carbohydrate and protein.

The present experiments were carried out in an attempt to get further light on the possibility of a synthesis of uric acid in the mammalian body. They were performed on the writer himself, 31 years of age, 65 kilos in weight and perfectly healthy. During the course of the experiments all excessive exercise was avoided. The diet consisted of purine-free food-stuffs and was consumed in three portions. The amount of fluid consumed was not measured but was approximately constant. Olive oil had to be substituted after the first diet for butter and cream as these food materials could not be taken by the subject on account of the diarrhoea which they induced.

The analytical methods employed were as follows: total nitrogen, Kjeldahl; ammonia, Folin; uric acid, Hopkins' method as described by Kennaway [1909]; purine bases, Camerer-Arnstein, after removal of uric acid according to Kennaway; acetone and acetoacetic acid by Messinger-Huppert. All the figures recorded are the means of closely agreeing duplicates.

Experiment I.

Diets consumed in different periods.

I *A*		I *B*		I *C*	
Dried skimmed milk	= 46 g.	Dried skimmed milk	= 105 g.	Dried skimmed milk	= 46 g.
Double cream	= 20 „	Water biscuit	= 100 „	Water biscuit	= 66 „
Butter	= 24 „	Olive oil	= 164 „	Caseinogen	= 27 „
Tapioca	= 117 „	NaCl	= 5 „	Olive oil	= 190 „
Water biscuit	= 341 „			NaCl	= 5 „
Cane sugar	= 20 „				
NaCl	= 5 „				
Protein	= 50 g.	Protein	= 50 g.	Protein	= 50 g.
Carbohydrate	= 443 „	Carbohydrate	= 137 „	Carbohydrate	= 80 „
Fat	= 30 „	Fat	= 165 „	Fat	= 190 „
Calorie intake	=2300 cal.	Calorie intake	=2300 cal.	Calorie intake	=2300 cal.

In the three periods of this experiment the protein intake and calorie value were kept approximately constant whilst the carbohydrate and fat content was varied. Period *A* was carbohydrate-rich, fat-poor; in *B* carbohydrate was largely, and in *C* almost entirely, replaced by fat. Between each period of the experiment a few days elapsed. The first day's urine of each period in this and in all subsequent experiments was discarded. The results of these diets on the composition of the urine are found in Tables I *A*, I *B*, and I *C*.

TABLE I *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
I	Acidic	7·53	0·46	0·110	0·008	0·118	6·11	1·46	0·11	1·57
II	„	8·26	0·46	0·108	0·012	0·120	5·57	1·31	0·15	1·46
III	„	7·79	0·43	0·093	0·011	0·104	5·52	1·19	0·14	1·33
IV	„	7·63	0·39	0·092	0·014	0·106	5·11	1·21	0·18	1·39
V	„	7·99	0·41	0·095	0·013	0·108	5·13	1·19	0·16	1·35
VI	„	7·63	0·39	0·089	0·016	0·105	5·11	1·17	0·21	1·38
Average		(7·81)	(0·42)	(0·098)	(0·012)	(0·110)	(5·43)	(1·26)	(0·16)	(1·41)

TABLE I *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
I	Acidic	7·71	0·45	0·095	0·012	0·106	5·84	1·23	0·16	1·39
II	„	7·56	0·37	0·087	0·018	0·105	4·89	1·15	0·24	1·39
III	Amphoteric	8·11	0·36	0·090	0·019	0·109	4·44	1·11	0·23	1·34
IV	„	8·50	0·29	0·095	0·014	0·109	3·41	1·12	0·16	1·28
V	„	8·60	0·25	0·097	0·018	0·115	2·91	1·13	0·21	1·34
VI	„	8·55	0·29	0·090	0·019	0·109	3·39	1·05	0·22	1·27
Average		(8·17)	(0·34)	(0·092)	(0·017)	(0·109)	(4·15)	(1·13)	(0·20)	(1·34)

TABLE I *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
I	Acidic	9·86	0·76	0·074	0·024	0·098	7·71	0·75	0·24	0·99
II	„	7·90	0·71	0·070	0·018	0·088	8·99	0·89	0·23	1·12
III	„	8·51	0·76	0·078	0·021	0·099	8·93	0·92	0·25	1·17
IV	„	8·32	0·67	0·071	0·017	0·088	8·05	0·85	0·20	1·05
V	„	9·25	0·96	0·057	0·024	0·081	10·38	0·62	0·26	0·88
Average		(8·77)	(0·77)	(0·070)	(0·021)	(0·091)	(8·81)	(0·81)	(0·24)	(1·04)

It will be noted that the reaction of the urine in period *B* is amphoteric from the third day and in this way differs from the urine of the first and third periods. This variation in reaction is almost certainly due to the fact that the diet of this period contained a large amount of dried skimmed milk, rich in alkaline salts. This was added to bring the protein content up to the proper level.

Total nitrogen. These three experiments demonstrate clearly the well-marked protein sparing action which the carbohydrates possess. In the carbohydrate-rich period *A*, the average nitrogen excretion is 7·81 g., in the second period it has increased slightly to 8·17 g. and in the fat-rich third period it stands at 8·77 g.

Ammonia. It is a well-known fact that if carbohydrate be taken in insufficient amount, particularly when the diet is rich in fat, acidosis results and this was of course found in this experiment. The reactions for acetone and acetoacetic acid were pronounced in the third period, but were completely absent in the first. Under these conditions it was to be expected that the output of ammonia would be lowest in period *A* and highest in *C* and be intermediate in amount in period *B*. As a matter of fact the average output in the first period was 0·42 g. N as ammonia, it fell to 0·34 g. in the second period and rose to 0·77 g. in the third. The explanation of the abnormal second period lies in the fact already mentioned that in period *B* dried skimmed milk was present in large amounts and the alkaline salts prevented any undue call on ammonia for neutralisation purposes.

Uric acid. In the first period there was a gradual fall in the output of uric acid during the course of the experiment from 0·11 g. on the first day to 0·089 g. on the sixth day, giving an average output of 0·098 g. In the second period the output was less varied, the average for the period being 0·092 g. In the third period the maximum output was 0·078 g. on the third day and the minimum 0·057 g. on the fifth day, giving an average of 0·070 g.

Thus it is found that the greatest excretion of uric acid was during the carbohydrate-rich first period and the lowest with the fat-rich diet. This effect is also clearly seen when the percentage figures are considered.

There is however one point which deserves some attention, namely that the output during the second period differs hardly at all from that during the first in spite of the great differences in carbohydrate content of the diet. This again must be ascribed to the presence of the alkali-rich dried milk and in support of such a contention there is the work of Macleod and Haskins [1906] who showed that the output of endogenous uric acid is increased by the ingestion of alkali. This point will be dealt with in another experiment in this paper. (Experiment III.)

Purine bases. The output of these bases in all three periods is not very constant. It is interesting to note that in the first period, in contradistinction to the uric acid, there is a gradual increase in the output of the basal purines. The average output for period *A* was 0·012 g. N as purine bases. In the other two periods there is no evidence of a progressive fall, the average excretion for the second period was 0·017 g. and for the third 0·021 g.

It will be observed that the purine base output reaches its lowest on the carbohydrate-rich first diet whilst the figure for the second period is about the mean value of the other two. This result agrees with the observation of Graham and Poulton who found that when carbohydrate was diminished in a diet, the purine base output showed a tendency to rise whilst the uric acid diminished.

The average values of the total purine output for the three periods were *A* 0·110 g., *B* 0·109 g. and *C* 0·091 g. N.

Experiment II.

In this experiment the effect of varying either the content of fat (Period *A*) or of carbohydrate (Period *B*) was tried, the content of protein and of carbohydrate or fat respectively being kept constant. As this variation necessitated a large variation in the calorie value of the diet, the duration of the individual periods of observation was reduced to two days so as to prevent invalidation of the experiment due to insufficient energy supply.

The total nitrogen and ammonia figures show nothing of note. As regards the uric acid the average value in the first two days was 0·068 g. and in the second 0·092 g. From these figures it is clear that when fat was withdrawn in large amount, notwithstanding the diminution in the energy

value of the diet, the uric acid output both in absolute amount and in percentage value of total nitrogen rose. This rather suggests that fat may have some inhibiting action on the uric acid output.

As regards the purine bases there is no appreciable difference between the outputs under the altered conditions.

Period A.

Diet during first two days		Diet during second two days	
Water biscuit	60 g.	Water biscuit	60 g.
Milk	500 cc.	Milk	500 cc.
Boiled egg white	120 g.	Boiled egg white ...	120 g.
Boiled egg yolk	80 „	Boiled egg yolk	80 „
Olive oil	147 „	NaCl	5 „
NaCl	5 „		
Protein	52 g.	Protein	52 g.
Carbohydrate	79 „	Carbohydrate	79 „
Fat	190 „	Fat	43 „
Calorie intake	2304 cal.	Calorie intake	937 cal.

TABLE II *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Test for aceto-acetic acid
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines	
I	Acidic	9·97	0·74	0·068	0·019	0·087	7·42	0·68	0·19	0·87	+
II	„	9·88	0·77	0·067	0·017	0·084	7·79	0·67	0·17	0·84	+
Average		(9·93)	(0·76)	(0·068)	(0·018)	(0·086)	(7·61)	(0·68)	(0·18)	(0·86)	
III	„	9·80	0·51	0·087	0·018	0·105	6·14	0·89	0·18	1·07	−
IV	„	10·30	0·54	0·097	0·016	0·113	5·24	0·94	0·16	1·10	−
Average		(10·05)	(0·53)	(0·092)	(0·017)	(0·109)	(5·69)	(0·92)	(0·17)	(1·09)	

In this series the protein and fat were kept constant but there was a variation of 259 g. of carbohydrate. Here the uric acid output fell with the diminution in the carbohydrate content from 0·111 g. to 0·096 g. N, a fact which is even more clearly demonstrated when the percentage figures are considered.

The result of both sets of experiments in *A* and *B* quite agree with the observation of Cathcart who found that the administration of fat after a period of fasting reduces the output of uric acid, whilst the administration of carbohydrate increases the output.

These observations are certainly very suggestive of a synthesis of uric acid in the organism as suggested by Wiener [1902] from lactic acid and urea. The urea is always present in considerable amount and lactic acid

is certainly one of the products which result from the catabolism of carbohydrate.

Period B.

Diet consumed during first two days		Diet consumed during second two days	
Water biscuit	160 g.	Water biscuit	100 g.
Milk	500 cc.	Milk	415 cc.
Rice	412 g.	Boiled egg white ...	120 g.
Cane sugar	36 „	Caseinogen	12 „
NaCl	5 „	NaCl	5 „
Protein	52 g.	Protein	52 g.
Carbohydrate	368 „	Carbohydrate	109 „
Fat	15 „	Fat	15 „
Calorie intake	1862 cal.	Calorie intake	800 cal.

TABLE II *B.*

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Test for aceto-acetic acid
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines	
I	Acidic	8·24	0·59	0·117	0·012	0·129	7·16	1·42	0·15	1·57	–
II	„	7·28	0·61	0·105	0·017	0·122	8·38	1·44	0·23	1·67	–
Average		(7·76)	(0·60)	(0·111)	(0·015)	(0·126)	(7·77)	(1·43)	(0·19)	(1·62)	
III	Amphoteric	8·20	0·45	0·100	0·017	0·117	5·49	1·22	0·21	1·43	–
IV	Acidic	9·25	0·47	0·091	0·014	0·105	5·08	0·98	0·15	1·13	–
Average		(8·73)	(0·46)	(0·096)	(0·016)	(0·111)	(5·29)	(1·10)	(0·18)	(1·28)	

Another experiment (II *C*) was carried out when the alteration in the carbohydrate content was made in a diet with a high intake of fat, and compared with the first set of figures of II *A*.

Diet during two days of Period *C*		Diet as in the first series of Period II *A*	
Milk	415 cc.		
Water biscuit	160 g.		
Rice	412 „		
Cane sugar	36 „		
Olive oil	175 „		
NaCl	5 „		
Protein	52 g.	Protein	52 g.
Carbohydrate	368 „	Carbohydrate	79 „
Fat	190 „	Fat	190 „
Calorie intake	3489 cal.	Calorie intake	2304 cal.

TABLE II *C.*

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Test for aceto acetic acid
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines	
I	Acidic	8·06	0·48	0·108	0·011	0·119	5·96	1·34	0·14	1·48	–
II	„	7·52	0·63	0·118	0·009	0·127	8·36	1·57	0·12	1·69	–
Average		(7·79)	(0·56)	(0·113)	(0·010)	(0·123)	(7·17)	(1·46)	(0·13)	(1·59)	

TABLE II *D*.

	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
	Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
Average values in the first series of Table II *A*	9·93	0·76	0·068	0·018	0·086	7·61	0·68	0·18	0·86
Average values of Table II *C*	7·79	0·56	0·113	0·010	0·123	7·17	1·46	0·13	1·59

It will be observed that there is a very decided difference between the total nitrogen output in the two sets of figures, this being much diminished on the carbohydrate-rich diet. There is also much less ammonia excreted with this diet. Again the uric acid output rises definitely, with the increase in the carbohydrate, from 0·068 g. to 0·113 g. N. This large rise, much more definite than in II *B*, is probably due to a synthesis but it may be also in part due to the disappearance of the acidosis.

There is also a marked difference between the output of the purine bases. Again it is found that the excretion of the bases is higher on the carbohydrate-poor than on the carbohydrate-rich diet.

Another interesting comparison may be made between the first series of II *B* and II *C* where the diets contained the same amounts of protein and carbohydrate but varied in their fat content.

Diet as in the first series of II *B*		Diet during II *C*	
Protein	52 g.	Protein	52 g.
Carbohydrate	368 „	Carbohydrate	368 „
Fat	15 „	Fat	190 „
Calorie intake	1862 cal.	Calorie intake	3489 cal.

TABLE II *E*.

	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
	Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines
Average values in the first series of Table II *B*	7·76	0·60	0·111	0·015	0·126	7·77	1·43	0·19	1·62
Average values of Table II *C*	7·79	0·56	0·113	0·010	0·123	7·17	1·46	0·13	1·59

As will be seen the alteration in the fat content of a diet rich in carbohydrate affects but little the composition of the urine except as regards the purine bases.

Experiment III.

This experiment was carried out for the purpose of testing the influence of alkali on the output of uric acid and purine bases on a fat-rich carbo hydrate-poor diet and on a fat-poor carbohydrate-rich one, the protein content and calorie value of the diets being kept approximately constant.

III *A*. Fat diet		III *B*. Carbohydrate diet	
Water biscuit	63 g.	Milk	500 cc.
Milk	500 cc.	Boiled egg white ...	120 g.
Boiled egg white ...	120 g.	Boiled egg yolk	80 „
Boiled egg yolk	80 „	Cane sugar	214 „
Olive oil	147 „	Rice	120 „
NaCl	5 „	Tapioca	160 „
		NaCl	5 „
Protein	52 g.	Protein	52 g.
Carbohydrate	79 „	Carbohydrate	413 „
Fat	190 „	Fat	43 „
Calorie intake	2304 cal.	Calorie intake	2306 cal.

TABLE III *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Acetoacetic acid g. reckoned as acetone	Test for acetoacetic acid	Remarks
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines			
I	Acidic	9·04	0·59	0·091	0·016	0·107	6·53	1·01	0·18	1·18	?	+	
II	„	8·40	0·62	0·070	0·016	0·086	7·38	0·83	0·19	1·02	0·103	+	
III	„	10·47	0·63	0·075	0·014	0·089	6·02	0·72	0·13	0·85	0·237	+	
IV	Alkaline	9·41	0·24	0·086	0·026	0·112	2·55	0·91	0·28	1·19	0·297	+	15 g. $NaHCO_3$ taken
V	„	9·41	0·12	0·121	0·017	0·138	1·28	1·29	0·18	1·47	0·305	++	
VI	„	8·99	0·16	0·099	0·017	0·116	1·78	1·10	0·19	1·28	0·395	++	
VII	Amphoteric	8·52	0·33	0·085	0·025	0·110	3·47	0·89	0·26	1·16	0·524	++	
VIII	Acidic	9·36	0·56	0·079	0·018	0·097	5·98	0·84	0·19	1·04	0·432	++	
IX	„	9·84	0·74	0·074	0·017	0·091	7·52	0·75	0·17	0·92	0·515	++	

TABLE III *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Acetoacetic acid g. reckoned as acetone	Test for acetoacetic acid	Remarks
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines			
I	Acidic	7·76	0·50	0·111	0·020	0·131	6·44	1·43	0·26	1·69	0	–	
II	„	7·22	0·52	0·095	0·021	0·116	7·20	1·32	0·29	1·61	0	–	
III	„	6·89	0·52	0·100	0·018	0·118	7·69	1·45	0·26	1·71	0	–	
IV	Alkaline	6·99	0·20	0·111	0·019	0·130	2·86	1·59	0·27	1·86	0	–	15 g. $NaHCO_3$ taken
V	„	6·01	0·09	0·113	0·012	0·125	1·56	1·88	0·20	2·08	0	–	
VI	„	6·14	0·09	0·121	0·016	0·137	1·47	1·97	0·26	2·23	0	–	
VII	Amphoteric	6·08	0·28	0·096	0·018	0·114	4·61	1·58	0·30	1·88	0	–	
VIII	Acidic	6·28	0·46	0·096	0·022	0·118	7·35	1·53	0·35	1·88	0	–	
IX	„	6·36	0·46	0·089	0·020	0·109	7·23	1·40	0·31	1·71	0	–	

In each series of experiments for three days (fourth to sixth day) 5 g. of sodium bicarbonate were taken thrice daily so that the urine was kept alkaline for this period.

Total nitrogen. There is no evidence of any marked influence of the alkali.

Ammonia. The administration of the bicarbonate causes a very definite fall in the amount of ammonia excreted. The average output on the non-alkali days in the case of the fat diet was 0·58 g. N and on the alkali days 0·17 g. N, in the case of the carbohydrate it fell from 0·46 g. to 0·13 g.

Uric acid. Haig [1887], Gorsky [1888] and Macleod and Haskins [1906] have all observed an increased output of uric acid after the administration of alkali. In the present series the variation between the two averages of uric acid output on the fat and carbohydrate diets, excluding the alkali days, is very definite: 0·077 g. N with the fat and 0·096 g. with the carbohydrate (average of five days omitting the first day in each experiment). As the result of giving bicarbonate the uric acid excretion rose in both sets of experiments; on the fat diet the average value for the three days was 0·102 g. N, an increase of 25 mg. over the output on non-alkali days, and on the carbohydrate diet the average value for the three days was 0·115 g., i.e. 19 mg. higher than the average output on the non-alkali days. Thus it would appear that the administration of an alkali does definitely increase the output of uric acid and it would further seem to be more effective in the case of the fat diet. This observation may afford the clue to the cause of the low output of uric acid on a fat diet when the carbohydrate supply is defective. The acidosis which results probably brings about a reduction in the alkalinity of the body fluids and either checks the output of uric acid or increases its chances of disintegration.

Macleod and Haskins in their experiments found that the increased output of purine continued for some days after the cessation of the administration of the alkali, but this increase was not observed in the present experiments.

Purine bases. There is no very definite evidence of any influence of the alkali on the output of the bases.

Experiment IV.

In this experiment the influence of glycerol on the output of uric acid and purines was to be tested on two diets, in one of which fat predominated and in the other carbohydrate. Unfortunately only the carbohydrate-rich

diet can be reported as the subject became ill during the course of the fat-rich diet.

Milk	500 cc.	Protein	79 g.
Rice	500 g.	Carbohydrate	425 „
Water biscuit	220 „	Fat	32 „
Boiled egg (white & yolk)	106 „	Calorie intake	2364 cal.
NaCl	5 „		

TABLE IV.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Aceto-acetic acid g. reckoned as acetone	Test for aceto-acetic acid	Remarks
		Total nitrogen	Ammonia	Uric acid	Purine bases	Total purines	Ammonia	Uric acid	Purine bases	Total purines			
I	Acidic	9·16	0·64	0·104	0·014	0·118	6·99	1·14	0·15	1·29	0	–	
II	„	9·30	0·66	0·108	0·018	0·126	7·11	1·16	0·19	1·35	0	–	
III	„	9·07	0·61	0·099	0·021	0·120	6·73	1·09	0·23	1·32	0	–	
IV	„	8·85	0·70	0·097	0·022	0·119	7·91	1·10	0·25	1·34	0	–	30 g. glycerol taken
V	„	8·43	0·77	0·126	0·013	0·139	9·13	1·49	0·15	1·64	0	–	50 g. „ „
VI	„	8·65	0·89	0·122	0·021	0·143	10·29	1·41	0·24	1·65	0	–	50 g. „ „
VII	„	8·60	0·72	0·127	0·018	0·145	8·37	1·48	0·21	1·69	0	–	
VIII	„	9·20	0·64	0·101	0·021	0·122	6·96	1·10	0·23	1·33	0	–	

30 g. of glycerol were taken on the fourth day and 50 g. on the fifth and sixth days.

There seems to be a slight reduction in the total nitrogen output as the result of the taking of glycerol, whereas in the case of the ammonia output, for some unknown reason, it seems to be quite definitely increased. The average output for the three pre-days is 0·64 g. N and for the three glycerol days 0·75 g. The effect is even more clearly seen when the percentage figures are considered where a rise from 6·94 % to 9·11 % occurs.

Uric acid. Horbaczewski and Kanera [1892] found that the output of uric acid was increased by the ingestion of glycerol, and this observation was confirmed in the present experiment. The average output for the three pre-days was 0·104 g. N and, although the output fell to 0·097 g. on the first glycerol day, the average for the three days was 0·115 g. or for the second and third days 0·124 g. The increase is also clearly seen in the percentage figures. It is possible that this increase is due to a synthesis of uric acid, the glycerol in the course of its catabolism giving rise to a certain amount of lactic acid.

Purine bases. The output of bases is apparently uninfluenced by the ingestion of glycerol.

PART II.

It is a well-known fact that the amount of uric acid in the urine of the dog is minimal, its place being taken by allantoin. This substance was found for the first time in normal dog's urine by Meissner [1868]. Salkowski [1876] was the first worker to show that the output of allantoin in the dog was increased by the administration of uric acid. Cohn [1898] obtained a similar result after feeding with thymus. Minkowski [1898] and Mendel and Brown [1900] have also confirmed this observation. Wiechowski [1907] obtained further confirmation by perfusing isolated organs with blood containing uric acid and obtaining allantoin. He stated [1908] that allantoin was to be considered as a normal end product of purine metabolism in the dog, cat, rabbit and monkey. Underhill and Kleiner [1908] showed that the allantoin present could be derived from endogenous purines as well as from exogenous. As regards the constancy of the daily excretion, this has been demonstrated by Wiechowski and by Hunter and Givens [1910].

As it was of interest to find whether carbohydrate had any influence on the amount of allantoin excreted, a series of experiments was carried out on a large Airedale bitch. The animal was kept in a suitable metabolism cage. She was catheterised at 10 A.M. each day and this urine was added to that collected in the cage. The methods employed were: total nitrogen, Kjeldahl; urea, Plimmer and Skelton's modification of urease method [1914]; ammonia, Folin; total purines, Camerer-Arnstein; allantoin by the method suggested by Plimmer and Skelton [1914].

The animal was fed on a purine- and allantoin-free diet. (Ackroyd [1911] states that milk contains a trace of allantoin but the amount is so small that the diet employed may be considered allantoin-free.)

Experiment V.

Diets employed:

A		*B*		*C*	
Oatmeal	70 g.	Oatmeal	30 g.	Oatmeal ...	30 g.
Margarine ...	80 „	Tapioca	100 „	Dry skimmed milk	30 „
Dry skimmed milk	30 „	Cane sugar ...	80 „	Caseinogen ...	7 „
NaCl	2 „	Dry skimmed milk	50 „	Margarine ...	100 „
		Margarine ...	10 „	NaCl	2 „
		NaCl	2 „		
Protein	22 g.	Protein	23 g.	Protein ...	23 g.
Carbohydrate ...	60 „	Carbohydrate ...	206 „	Carbohydrate ...	34 „
Fat	73 „	Fat	12 „	Fat	86 „
Calorie intake ...	1015 cal.	Calorie intake ...	1051 cal.	Calorie intake ...	1034 cal.

In the three periods of this diet the protein content was kept approximately constant whilst the carbohydrate-fat content was varied. The animal consumed its food in periods *A* and *B* with avidity but in period *C* (fat-rich, carbohydrate-poor diet) after the sixth day it always left some in the dish; it also developed mild diarrhoea. Accordingly in Table V*C* only the analyses for the first five days, which were free from objection, are recorded.

TABLE V *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·72	1·72	0·15	0·018	0·26	63·24	5·51	0·66	9·56
II	,,	2·32	1·65	0·19	0·020	0·27	71·12	8·19	0·86	7·33
III	,,	2·87	1·95	0·32	0·021	0·22	67·94	11·15	0·73	7·67
IV	,,	2·94	1·93	0·27	0·019	0·24	65·65	9·18	0·65	8·16
V	,,	2·87	1·78	0·29	0·016	0·35	62·02	10·10	0·56	12·20
VI	,,	2·78	1·71	0·31	0·010	0·31	61·51	10·43	0·36	11·15
VII	,,	2·62	1·48	0·18	0·012	0·23	56·49	11·83	0·46	8·78
VIII	,,	2·62	1·71	0·24	0·017	0·31	65·27	6·87	0·65	11·83
IX	,,	2·67	1·66	0·24	0·016	0·32	62·17	8·99	0·60	11·99
X	,,	2·73	1·70	0·31	0·013	0·21	62·27	11·36	0·48	7·69
Average		(2·71)	(1·73)	(0·26)	(0·016)	(0·26)	(64·77)	(9·36)	(0·60)	(9·64)

TABLE V *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·21	1·32	0·19	0·017	0·39	59·72	8·60	0·77	17·65
II	,,	2·38	1·27	0·33	0·027	0·40	53·36	13·87	1·22	16·81
III	,,	1·95	1·33	0·19	0·018	0·38	68·21	9·74	0·92	19·49
IV	,,	2·33	1·31	0·38	0·019	0·30	56·22	16·31	0·82	12·88
V	,,	2·38	1·47	0·26	0·022	0·37	61·76	10·92	0·92	15·55
VI	,,	1·66	1·28	0·38	0·023	0·33	77·11	22·82	1·39	19·88
VII	,,	2·07	1·24	0·22	0·018	0·29	59·90	10·63	0·87	14·01
VIII	,,	2·20	1·26	0·28	0·024	0·41	57·27	12·73	1·09	18·64
IX	,,	2·12	1·24	0·25	0·023	0·33	58·49	11·79	1·08	15·57
X	,,	2·20	1·40	0·22	0·022	0·34	63·64	10·00	1·00	15·45
Average		(2·15)	(1·31)	(0·27)	(0·021)	(0·35)	(61·77)	(12·75)	(1·01)	(16·59)

TABLE V *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·57	1·70	0·27	0·016	0·16	66·15	10·51	0·62	6·23
II	,,	2·73	1·89	0·19	0·017	0·17	69·23	6·96	0·62	6·23
III	,,	2·30	1·55	0·23	0·016	0·24	67·39	10·00	0·70	10·43
IV	,,	2·64	1·85	0·21	0·019	0·22	70·76	7·95	0·72	8·33
V	,,	2·79	1·96	0·19	0·018	0·20	70·25	6·81	0·65	7·17
Average		(2·61)	(1·79)	(0·22)	(0·017)	(0·20)	(68·76)	(8·45)	(0·66)	(7·68)

Reaction of urine. Throughout all three periods the reaction remained amphoteric.

Total nitrogen. Here again the protein-sparing action of the carbohydrate is clearly demonstrated, the output is lowest in V *B*. The same result is found in the case of the urea, the lowest amount excreted being on the carbohydrate-rich diet. The effect is seen very clearly in the percentage figures.

Ammonia. Throughout all three periods the output of ammonia tended to be rather irregular. But one point is quite clear, namely the ammonia output on the fat-rich diet gives no evidence of any acidosis. It is of course well known that it is difficult to induce an acidosis in a dog.

Purines. There is certainly a slightly increased output of basal purine in the carbohydrate-rich diet (V *B*) but the increase is not very great.

Allantoin. This substance forms the greatest part of the products of purine metabolism excreted by the dog, and as will be seen from the Tables V *A*, *B* and *C* the amount excreted is apparently dependent on the amount of carbohydrate present in the diet. The output is highest in the case of the carbohydrate-rich diet and is lowest in the carbohydrate-poor, being 0·35 g. nitrogen in V *B*, 0·26 g. in V *A*, and 0·20 g. in V *C*. This variation is seen even more distinctly when the percentage figures are examined: 16·59 %, 9·64 % and 7·68 % respectively. In other words it would seem that the allantoin output in dog's urine behaves just as the uric acid in the human urine. In the case of the dog the question of the part played by acidosis need not be considered, the increased output of allantoin being ascribed solely to the variation in the carbohydrate intake.

Experiment VI.

This experiment was carried out for the purpose of confirming the previous one.

Diets employed:

A		*B*		*C*	
Oatmeal	30 g.	Oatmeal	40 g.	Oatmeal	70 g.
Dry skimmed milk	30 „	Dry skimmed milk	30 „	Dry skimmed milk	30 „
Caseinogen ...	9 „	Tapioca	98 „	Caseinogen ...	3 „
Margarine ...	99 „	Margarine ...	50 „	Margarine ...	81 „
NaCl	2 „	Caseinogen ...	7 „	NaCl	2 „
		NaCl	2 „		
Protein	25 g.	Protein	25 g.	Protein	25 g.
Carbohydrate ...	34 „	Carbohydrate ...	125 „	Carbohydrate ...	60 „
Fat	85 „	Fat	45 „	Fat	74 „
Calorie intake ...	1032 cal.	Calorie intake ...	1034 cal.	Calorie intake ...	1037 cal.

TABLE VI *A*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	3·04	2·35	0·36	0·026	0·10	77·30	11·84	0·86	3·29
II	,,	3·04	2·02	0·52	0·022	0·16	68·42	17·11	0·72	5·26
III	,,	2·86	1·99	0·36	0·019	0·12	69·58	12·59	0·66	4·20
IV	,,	2·92	1·99	0·38	0·021	0·18	68·15	13·01	0·72	6·16
V	,,	2·76	1·85	0·39	0·018	0·14	67·03	14·13	0·65	5·07
Average		(2·92)	(2·04)	(0·40)	(0·021)	(0·14)	(70·10)	(13·74)	(0·72)	(4·80)

TABLE VI *B*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·55	1·39	0·33	0·016	0·34	54·51	12·94	0·63	13·33
II	,,	2·24	1·44	0·30	0·021	0·30	64·29	13·39	0·94	13·39
III	,,	2·21	1·54	0·27	0·013	0·27	69·68	12·22	0·59	12·22
IV	,,	2·44	1·78	0·34	0·012	0·32	72·95	13·93	0·49	13·11
V	,,	2·63	1·53	0·30	0·012	0·29	58·17	11·41	0·46	11·03
Average		(2·41)	(1·54)	(0·31)	(0·015)	(0·30)	(63·92)	(12·78)	(0·62)	(12·62)

TABLE VI *C*.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of			
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin
I	Amphoteric	2·70	2·03	0·30	0·015	0·22	75·19	11·11	0·56	8·15
II	,,	2·54	1·93	0·20	0·019	0·18	75·98	7·87	0·75	7·09
III	,,	2·49	1·42	0·30	0·015	0·23	57·03	12·05	0·60	9·24
IV	,,	2·60	1·62	0·23	0·015	0·22	62·31	8·85	0·58	8·46
V	,,	2·98	2·24	0·29	0·016	0·26	75·17	9·73	0·54	8·72
Average		(2·66)	(1·85)	(0·26)	(0·016)	(0·22)	(69·14)	(9·23)	(0·61)	(8·33)

The outputs of total nitrogen and urea agree with the previous results, but for some reason the output of ammonia on the carbohydrate-rich diet VI *B* is higher than on VI *C*. In the case of the purine bases the output is highest on the fat-rich diet and no conclusion regarding the influence of food on the output of bases can be reached.

Allantoin. The result here is similar to that in the previous experiment, i.e. the output is greatest with the diet containing most carbohydrate.

Experiment VII.

It has been clearly shown by Minkowski [1886], Wiener [1901], Kowalewski and Salaskin [1901] and others that in the avian organism at least it can be readily demonstrated that uric acid is formed by the condensation of lactic acid with urea, and Wiener has maintained that the same process can take place in the mammalian organism. All previous experiments so far as I am aware have been based on the assumption that uric acid was an end product, and in this experiment attention was directed to the influence of the ingestion of lactic acid on the output of allantoin, the normal end product of purine metabolism in the dog.

A fat-rich carbohydrate-poor diet was selected, as the normal allantoin output would be low and thus the influence of the ingested lactic acid could be more readily detected.

Diet:

Oatmeal	30 g.	Protein	25 g.
Dry skimmed milk	30 „	Carbohydrate	34 „
Caseinogen	9 „	Fat	84 „
Margarine	100 „	Calorie intake	1032 cal.
NaCl	2 „		

TABLE VII.

Day of experiment	Reaction of urine on litmus	Excretion of nitrogen in g. of					Percentage of total nitrogen in form of				Remarks
		Total nitrogen	Urea	Ammonia	Total purines	Allantoin	Urea	Ammonia	Total purines	Allantoin	
I	Amphoteric	2·84	1·90	0·39	0·022	0·18	66·90	13·73	0·77	6·34	
II	„	3·05	1·73	0·31	0·022	0·23	56·72	10·16	0·72	7·54	
III	„	3·31	2·23	0·25	0·021	0·20	67·37	7·55	0·63	6·04	
Average		(3·07)	(1·95)	(0·32)	(0·022)	(0·20)	(63·66)	(10·48)	(0·70)	(6·64)	
IV	„	2·73	1·92	0·27	0·019	0·30	70·33	9·89	0·70	10·99	10 g. sod. lactate
V	„	2·80	2·09	0·25	0·020	0·25	74·64	8·93	0·71	8·93	„ „
VI	„	2·67	1·83	0·25	0·021	0·31	68·54	9·36	0·79	11·61	„ „
Average		(2·73)	(1·95)	(0·26)	(0·020)	(0·28	(71·17)	(9·39)	(0·73)	(10·51)	
VII	„	2·60	1·77	0·30	0·020	0·25	68·08	11·54	0·77	9·62	
VIII	„	2·48	1·74	0·29	0·016	0·15	70·16	11·69	0·65	6·05	
IX	„	2·42	1·76	0·32	0·016	0·13	72·73	13·22	0·66	5·37	
Average		(2·50)	(1·76)	(0·30)	(0·017)	(0·18)	(70·32)	(12·15)	(0·69)	(7·03)	
X	„	2·46	1·58	0·28	0·016	0·36	64·23	11·38	0·61	14·63	20 g. sod. lactate
XI	„	2·55	1·72	0·32	0·018	0·33	67·45	12·55	0·71	12·94	„ „
XII	„	2·79	2·05	0·30	0·017	0·27	73·48	10·75	0·61	9·68	„ „
Average		(2·60)	(1·78)	(0·30)	(0·017)	(0·32)	(68·39)	(11·56)	(0·64)	(12·42)	
XIII	„	2·73	1·76	0·28	0·017	0·18	64·47	10·26	0·62	6·59	
XIV	„	2·65	1·63	0·35	0·017	0·18	61·51	13·21	0·64	6·79	
Average		(2·69)	(1·70)	(0·32)	(0·017)	(0·18)	(62·99)	(11·74)	(0·63)	(6·69)	

On the mornings of the fourth, fifth and sixth days 10 g. of sodium lactate and on the tenth, eleventh and twelfth days 20 g. of sodium lactate were given.

The ingestion of the sodium lactate is apparently without influence on the output of total nitrogen, urea, ammonia and purine bases.

Allantoin. In the case of this substance the administration of the sodium lactate seems to exert a very direct influence. The average output during the first three normal days was 0·20 g. allantoin nitrogen, this rose to an average output of 0·29 g. after the giving of 10 g. sodium lactate; on the three following normal days the output declined to an average of 0·18 g. and rose again to 0·32 g. on giving 20 g. of sodium lactate. The output on the two following normal days returned to its old level of 0·18 g. The result is also clearly seen when the figures of the percentage of total nitrogen are considered. Although, then, the actual amounts of allantoin excreted are not perhaps very large they show a definite increase following the ingestion of sodium lactate and it would therefore seem probable that an actual synthesis of uric acid had taken place.

Conclusions.

1. The protein-sparing action of carbohydrate as compared with fat is again clearly demonstrated in this series of experiments. Glycerol would seem also to act as a protein sparer.

2. In the case of man there is some evidence of the synthetic formation of uric acid when the diet is rich in carbohydrate. In the dog the same result is obtained in the case of allantoin.

3. The output of endogenous uric acid in the human urine is increased after the ingestion of glycerol.

4. There is a distinct rise in the output of allantoin in the dog's urine after the administration of sodium lactate.

5. The output of endogenous uric acid is increased by the giving of sodium bicarbonate.

6. In several experiments it was found that a decrease in the output of purine bases was associated with an increase in the excretion of uric acid.

7. In fat-rich diets which are carbohydrate-poor the output of uric acid is markedly diminished.

This investigation was carried out at the suggestion and under the direction of Dr E. P. Cathcart, to whom I offer my thanks. The expenses were defrayed in part by a grant from the Carnegie Trust made to Dr Cathcart.

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XLII. THE INFLUENCE OF CARBOHYDRATE AND FAT ON PROTEIN METABOLISM WITH SPECIAL REFERENCE TO THE OUTPUT OF SULPHUR.

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(*Received October 20th, 1915.*)

In view of the fact that carbohydrate brings about a very definite reduction in the output of nitrogen in the urine and that fat tends to raise this output, when carbohydrate is present in small amount, it was of interest to find how the sulphur output would behave under similar conditions. The N : S ratio has now been repeatedly investigated, with special reference to the rate at which these two substances are excreted [Siven, 1901; Sherman and Hawk, 1901; von Wendt, 1905; Ehrström, 1906; Falta, 1906; Hämäläinen and Helme, 1907; Wolf, 1912; and Cathcart and Green, 1913]. These observers however do not all agree in their conclusions, some maintain that the nitrogen and sulphur are excreted at a parallel rate, whilst others hold that the sulphur appears before the nitrogen. Whatever the rate, the general conclusion of von Wendt is well founded, viz. that the only true picture of the total protein exchange in the body is obtained when the nitrogen and sulphur excretions are considered together.

As regards the partition of the sulphur present in the urine, Folin's [1905] conclusions may be accepted. He found that when the total sulphur excretion falls, the percentage of sulphur present as inorganic sulphates diminishes—these bear almost the same relationship to total sulphur as urea does to total nitrogen, i.e. they represent the products which are chiefly involved in quantitative changes of the total sulphur. The ethereal sulphates and neutral sulphur would seem to have an origin analogous to that of purines and creatinine. Folin found that the ethereal sulphate output becomes

more prominent when the food contains little or no protein and that the neutral sulphur represents products "which in the main are independent of the total amount of sulphur eliminated or of protein katabolised."

The method employed in the present experiment was that of superimposition, i.e. on a particular day there was added to a standard diet the special foodstuff to be tested, the content of which in nitrogen and sulphur was known. In different cases the diet was rich in fat and correspondingly poor in carbohydrate, or fat-poor and carbohydrate-rich. As the subject of the experiment a healthy bitch weighing approximately 13 kilo. was employed. The animal was kept in a metabolic cage in the usual way. She was catheterised each morning at ten and the urine added to that collected in the receiver of the cage. The urine, after the quantity, reaction, and specific gravity had been determined, was always made up to 800 cc. with water.

The diets employed were as follows:

Carbohydrate-rich		Fat-rich	
Oatmeal	70 g.	Oatmeal	70 g.
Tapioca	100 g.	Dry skimmed milk	30 g.
Cane sugar	40 g.	Margarine	90 g.
Dry skimmed milk	30 g.	Water	500 cc.
Margarine	20 g.		
Water	500 cc.		

The animal was allowed to have drinking water *ad libitum*.

The methods of analysis employed were: total nitrogen, Kjeldahl; total sulphur, Benedict; inorganic and ethereal sulphates, Folin. The faeces were not examined.

The standard diet was continued until the output of total nitrogen and total sulphur became fairly regular. Then an amount of the protein to be tested, which contained approximately 6 g. of nitrogen, was added to the standard diet at the usual feeding time. The standard diet was then continued until the output of total nitrogen and of total sulphur was again regular. The extra amounts of nitrogen and sulphur excreted were determined by the subtraction of the average amount of nitrogen and sulphur excreted on the three days immediately preceding the superimposition from that excreted after the addition.

Experiment I. Addition of Caseinogen.

The amount of caseinogen was 50 g., containing 6·21 g. total nitrogen, 0·482 g. of total sulphur (0·179 g. in the form of oxidised sulphur), thus giving an S : N ratio of 1 : 12·8. The result of this superimposition in the case of the two diets is seen in the following tables.

TABLE I. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
v	470	acid	1010	2·43	0·166	0·079	0·012	0·075	47·6	7·2	45·2	1 : 14·6	
vi	520	amph.	1009	2·41	0·166	0·071	0·013	0·082	42·8	7·8	49·4	1 : 14·5	
vii	540	acid	1008	2·44	0·168	0·074	0·011	0·083	44·0	6·5	49·4	1 : 14·5	
viii	560	,,	1011	4·00	0·241	0·150	0·013	0·078	62·2	5·5	31·3	1 : 16·6	50 g. caseinogen
ix	640	,,	1006	2·54	0·187	0·092	0·013	0·083	48·9	6·8	44·3	1 : 13·6	
x	580	,,	1009	2·21	0·163	0·075	0·009	0·080	45·6	5·4	49·0	1 : 13·8	
xi	600	,,	1008	2·16	0·160	0·072	0·009	0·079	44·8	5·9	49·3	1 : 13·5	
xii	630	amph.	1006	2·18	0·146	0·064	0·011	0·071	43·6	7·5	48·8	1 : 15·0	
xiii	630	acid	1006	2·16	0·150	0·066	0·011	0·072	44·4	7·6	48·0	1 : 14·5	

TABLE II. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
iv	440	acid	1013	2·42	0·151	0·064	0·015	0·072	42·6	9·6	47·9	1 : 16·0	
v	640	amph.	1008	2·54	0·160	0·072	0·016	0·072	44·7	10·1	45·2	1 : 15·9	
vi	550	acid	1010	2·59	0·145	0·063	0·013	0·068	43·6	9·1	47·3	1 : 17·9	
vii	1040	,,	1007	5·55	0·341	0·233	0·018	0·089	68·5	5·3	26·2	1 : 16·3	50 g. caseinogen
viii	850	,,	1005	2·99	0·178	0·090	0·015	0·073	50·6	8·4	41·0	1 : 16·8	
ix	570	,,	1012	2·64	0·168	0·080	0·014	0·073	47·8	8·4	43·8	1 : 15·8	
x	530	,,	1012	2·22	0·129	0·054	0·012	0·063	41·7	9·6	48·7	1 : 17·2	
xi	480	,,	1011	2·42	0·143	0·061	0·015	0·066	43·1	10·5	46·5	1 : 17·0	

The extra-elimination of the nitrogen and the sulphur is shown in Tables I *A* and II *A*.

TABLE I *A*. (*Carbohydrate diet.*)

Day	T.N. in g.	S. in g.				T.S. : T.N.
		T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	
Fed day	1·57	0·074	0·075	0·001	0·002	1 : 21·1
First day	0·11	0·020	0·017	0·001	0·003	1 : 5·5
Total	1·68	0·094	0·092	0·002	0·005	1 : 17·9

TABLE II *A*. (*Fat diet.*)

Day	T.N. in g.	S. in g.				T.S. : T.N.
		T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	
Fed day	3·03	0·189	0·167	0·003	0·018	1 : 16·0
First day	0·47	0·026	0·024	—	0·002	1 : 18·8
Second day	0·12	0·016	0·014	0·001	0·002	1 : 7·5
Total	3·62	0·231	0·205	0·004	0·022	1 : 15·6

There is a definite rise in the output of total nitrogen and total sulphur after the superimposition, most marked on the day of feeding on both diets, and to a slight extent on the first day after with the carbohydrate diet, and on the first and second on the fat diet. This excess forms only 27 % of the nitrogen ingested with the carbohydrate diet: thus there is well-marked retention. But the retention is even more definite when the sulphur moiety is considered, as in the two days there is an excretion of only 0·094 g. total sulphur, i.e. 19·5 % of the sulphur ingested. When the fat diet is considered the results are different, as in the case of the nitrogen 58 % of that ingested is excreted and 48 % of the sulphur. In each instance the bulk of the sulphur excreted is in the form of inorganic sulphates.

As regards the other question, as to whether the carbohydrate-rich or fat-rich diet affects the distribution of the sulphur, there is no doubt about the fact that in this experiment when the outputs on the two diets are compared, although the total nitrogen output is slightly lower on the carbohydrate diet, the output of total sulphur is somewhat higher. As regards the partition of the sulphur it is of interest to note that on both diets about half of the sulphur excreted is in the form of neutral sulphur. Taking the mean of the three pre-days, it is found on the carbohydrate diet that 44·8 % is in the form of inorganic sulphates, 7·2 % as ethereal sulphates and 48·0 % as neutral sulphur. On the fat diet 43·6 % is present as inorganic sulphates, 9·6 % as ethereal sulphates, and 46·8 % as neutral sulphur. There is a slight

increase both in absolute and percentage amount in the output of ethereal sulphates on the fat diet.

Experiment II. Egg albumin.

50 g. of finely ground egg albumin containing 6·14 g. of nitrogen and 0·671 g. of total sulphur (with 0·041 g. in the oxidised form) were superimposed on the two standard diets as in the previous experiment. Unfortunately the fat diet could not be carried on until the old level was reached as the dog after the twelfth day refused to eat the food. The results are found in Tables III and IV.

The extra-elimination of the nitrogen and the sulphur is shown in Tables III *A* and IV *A*.

In the present experiment there is quite a marked difference between the output of the extra nitrogen and sulphur on the carbohydrate and fat diets. It required in the case of the carbohydrate-rich diet three days for the excretion of the excess nitrogen, 41·5 % of that ingested being turned out in this time, whereas the excretion of the sulphur continued quite definitely for seven days, 49·5 % of that ingested being excreted. With the fat diet this disparity of excretion was not found although the excretion of the nitrogen was continued over five days, with the result that 56·7 % of that ingested was turned out. In the case of the sulphur this also continued for five days, 47·4 % of that ingested being excreted.

Here then again, just as in the case of the caseinogen, the conditions for the retention of nitrogen on a carbohydrate diet are better than in the case of a fat diet, a difference of 31 % with the caseinogen and over 15 % with the egg albumin.

As in the previous experiment, the great bulk of the sulphur excreted is in the form of inorganic sulphates.

In this experiment not only is the total nitrogen output on the carbohydrate diet quite definitely lower but so is also the total sulphur output. The ratio of the output of sulphur to nitrogen in spite of these differences is practically identical in both experiments. It is therefore simply a clear case of complete reduction of the total protein catabolism.

When the form in which the sulphur is excreted is considered it will be noted that although on both diets the output is practically distributed between the inorganic sulphates and the neutral sulphur, in the case of the carbohydrate diet the greater part is in the form of neutral sulphur, whereas on the fat diet the greater part is present as inorganic sulphates.

TABLE III. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g. T.S.	S. in g. Inorganic sulphate	S. in g. Ethereal sulphate	S. in g. Neutral sulphur	Per cent. of T. sulphur Inorganic sulphate	Per cent. of T. sulphur Ethereal sulphate	Per cent. of T. sulphur Neutral sulphur	T.S. : T.N.	Remarks
vii	570	amph.	1009	2·35	0·126	0·051	0·010	0·065	40·5	7·9	51·6	1 : 18·6	
viii	610	„	1008	2·33	0·133	0·056	0·012	0·065	42·1	9·0	48·9	1 : 17·5	
ix	500	„	1009	2·33	0·130	0·057	0·010	0·063	43·8	7·7	48·5	1 : 17·9	
x	550	„	1014	4·37	0·273	0·188	0·018	0·067	68·9	6·6	24·5	1 : 16·0	50 g. egg albumin
xi	560	acid	1010	2·74	0·214	0·123	0·018	0·073	57·5	8·4	34·1	1 : 12·8	
xii	670	amph.	1008	2·45	0·171	0·088	0·015	0·068	51·5	8·8	39·8	1 : 14·3	
xiii	530	„	1011	2·24	0·150	0·067	0·012	0·071	44·7	8·0	47·3	1 : 14·9	
xiv	590	„	1007	2·32	0·150	0·069	0·014	0·067	46·0	9·3	44·7	1 : 15·5	
xv	590	„	1008	2·27	0·144	0·066	0·012	0·066	45·8	8·3	45·8	1 : 15·8	
xvi	610	acid	1011	2·37	0·140	0·064	0·013	0·063	45·7	9·3	45·0	1 : 17·1	
xvii	710	amph.	1006	2·37	0·130	0·055	0·013	0·062	42·3	10·0	47·7	1 : 18·2	

TABLE IV. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g. T.S.	S. in g. Inorganic sulphate	S. in g. Ethereal sulphate	S. in g. Neutral sulphur	Per cent. of T. sulphur Inorganic sulphate	Per cent. of T. sulphur Ethereal sulphate	Per cent. of T. sulphur Neutral sulphur	T.S. : T.N.	Remarks
v	490	alk.	1014	2·67	0·163	0·088	0·011	0·064	54·0	6·7	39·3	1 : 16·4	
vi	680	„	1009	2·52	0·145	0·069	0·011	0·065	47·6	7·6	44·8	1 : 17·4	
vii	740	„	1007	2·60	0·136	0·063	0·010	0·063	46·3	7·4	46·3	1 : 19·1	
viii	1050	amph.	1007	4·20	0·275	0·174	0·014	0·087	63·3	5·1	31·6	1 : 15·3	50 g. egg albumin
ix	690	alk.	1010	3·08	0·262	0·161	0·015	0·086	61·5	5·7	32·8	1 : 11·8	
x	960	amph.	1006	2·90	0·181	0·094	0·011	0·076	51·9	6·1	42·0	1 : 16·0	
xi	1100	„	1007	3·25	0·176	0·093	0·010	0·073	52·8	5·7	41·5	1 : 18·5	
xii	1000	„	1005	3·05	0·164	0·080	0·012	0·072	48·8	4·3	43·9	1 : 18·6	

TABLE III *A*. (*Carbohydrate diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	2·03	0·143	0·133	0·007	0·003	1 : 14·2
First „	0·40	0·084	0·068	0·007	0·009	1 : 4·8
Second „	0·11	0·041	0·033	0·004	0·004	1 : 2·7
Third „	—	0·020	0·012	0·001	0·007	—
Fourth „	—	0·020	0·014	0·003	0·003	—
Fifth „	—	0·014	0·011	0·001	0·002	—
Sixth „	—	0·010	0·009	0·002	−0·001	—
Total	2·54	0·332	0·280	0·025	0·027	1 : 7·6

TABLE IV *A*. (*Fat diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	1·60	0·127	0·101	0·003	0·023	1 : 12·6
First „	0·48	0·114	0·088	0·004	0·022	1 : 4·2
Second „	0·30	0·033	0·021	—	0·012	1 : 9·1
Third „	0·65	0·028	0·020	−0·001	0·009	1 : 23·2
Fourth „	0·45	0·016	0·007	0·001	0·008	1 : 28·1
Total	3·48	0·318	0·237	0·007	0·074	1 : 10·9

Experiment III. Gelatin.

As in both of the previous experiments the extra output of total nitrogen was higher on the fat than on the carbohydrate diet, i.e. more perfect retention having taken place on the latter diet, it was of some interest to see whether when an "imperfect" protein like gelatin was employed a similar disparity would occur. In this experiment 40 g. of gelatin containing 5·86 g. of nitrogen and 0·541 g. of sulphur (0·109 in an oxidised form) were dissolved in water and superimposed on the standard diets (Tables V and VI).

The extra-elimination of the nitrogen and the sulphur is shown in Tables V *A* and VI *A*.

As in the other experiments there is again a greater output of the superimposed nitrogen in the case of the fat diet; on the carbohydrate diet 78·3 % of the gelatin nitrogen ingested reappeared in the urine in the course of three days, and on the fat diet within the same period 88·7 % reappeared. In the case of the sulphur the result was very similar, on the carbohydrate diet 24·7 % and on the fat diet 30·3 % was excreted, in both instances the extra output, mainly in the form of inorganic sulphates, was confined to the fed-day.

TABLE V. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
iv	930	amph.	1008	2·03	0·141	0·056	0·015	0·070	39·7	10·6	49·6	1 : 14·4	
v	820	,,	1009	2·04	0·137	0·051	0·017	0·069	37·2	12·4	50·4	1 : 14·9	
vi	1050	,,	1007	2·16	0·141	0·058	0·016	0·067	41·1	11·3	47·5	1 : 15·3	
vii	1000	,,	1009	5·94	0·274	0·170	0·016	0·088	62·0	5·9	32·1	1 : 21·7	40 g. gelatin
viii	670	,,	1010	2·60	0·118	0·039	0·013	0·066	33·1	11·0	55·9	1 : 22·0	
ix	950	,,	1007	2·29	0·126	0·043	0·016	0·067	34·1	12·7	53·2	1 : 18·2	
x	730	,,	1008	2·08	0·144	0·060	0·017	0·067	41·7	11·8	46·5	1 : 14·4	
xi	790	,,	1008	2·18	0·153	0·071	0·015	0·067	46·4	9·8	43·8	1 : 14·3	

TABLE VI. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	T.N. in g.	S. in g.				Per cent. of T. sulphur			T.S. : T.N.	Remarks
					T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	Inorganic sulphate	Ethereal sulphate	Neutral sulphur		
ii	800	amph.	1009	2·32	0·145	0·062	0·013	0·070	42·8	9·0	48·3	1 : 16·0	
iii	700	,,	1010	2·29	0·157	0·070	0·016	0·071	44·6	10·2	45·2	1 : 14·5	
iv	1100	,,	1005	2·54	0·162	0·071	0·016	0·075	43·8	9·9	46·3	1 : 15·7	
v	1360	acid	1010	6·68	0·319	0·213	0·018	0·088	66·8	5·6	27·6	1 : 20·9	40 g. gelatin
vi	850	amph.	1007	3·06	0·140	0·057	0·015	0·068	40·7	10·7	48·6	1 : 21·9	
vii	1000	,,	1006	2·60	0·149	0·063	0·015	0·071	42·3	10·1	47·7	1 : 17·4	
viii	1000	acid	1006	2·23	0·135	0·051	0·014	0·070	37·8	10·4	51·9	1 : 16·5	

TABLE V *A*. (*Carbohydrate diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	3·86	0·134	0·115	—	0·019	1 : 28·8
First ,,	0·52	—	—	—	—	—
Second ,,	0·21	—	—	—	—	—
Total	4·59	0·134	0·115	—	0·019	1 : 34·3

TABLE VI *A*. (*Fat diet.*)

Day	T.N. in g.	S. in g. T.S.	Inorganic sulphate	Ethereal sulphate	Neutral sulphur	T.S. : T.N.
Fed day	4·30	0·164	0·145	0·003	0·016	1 : 26·2
First ,,	0·68	—	—	—	—	—
Second ,,	0·22	—	—	—	—	—
Total	5·20	0·164	0·145	0·003	0·016	1 : 31·7

It is of interest to note that the output of extra total nitrogen after the gelatin feeding is very definitely higher than is the case with the more "perfect" proteins caseinogen and egg albumin. It is difficult to say why the retention of sulphur is more marked than in the other experiments. It is of course possible that the standard diet, on which the animal was mainly living, was poor in sulphur and that there was a real demand for this substance. It must not be forgotten that in all probability the demand for sulphur by an animal like the dog is relatively greater than in the case of man, due to the fact that there is always a very steady loss of sulphur-rich material like hair.

These results certainly do not agree with many previous experiments, which however were carried out on human beings, where it has been practically uniformly found that if the output of sulphur did not actually exceed the total nitrogen, it at the very least ran parallel with it.

As regards the output of nitrogen and sulphur on the standard diet in this experiment, just as in Exp. II on the carbohydrate diet, the output of both nitrogen and sulphur is lower than on the fat diet. With respect to the partition of the sulphur on both diets, neutral sulphur is the most predominant constituent, although in both ethereal sulphates are excreted in greater amount than in either of the other two experiments.

These experiments not only bear out the statement first put forward by Falta and since confirmed by others that the rate of the excretion of the catabolic products of protein depends on the nature of the protein ingested,

but they allow of the further amplification that the rate at which these products are excreted depends not only on the nature of the protein but also on the nature of the diet on which the protein has been superimposed.

These experiments were carried out at the suggestion and under the direction of Dr E. P. Cathcart, to whom I offer my thanks.

The expenses were in part defrayed by a grant made to Dr Cathcart by the Carnegie Trust.

Summary.

1. In all three experiments the retention of superimposed nitrogen is greater on a carbohydrate than on a fat diet.

2. In two experiments out of the three the same holds good for sulphur.

3. The amount of extra nitrogen and sulphur excreted varies with the protein used.

4. The greater part of the extra sulphur excreted is in the form of inorganic sulphates.

5. The partition of sulphur on the carbohydrate and fat diets is very similar—it is for the most part excreted in the form of inorganic sulphates and neutral sulphur in almost equal amount.

6. There is no evidence that the protein retained after superimposition is poor in sulphur. Indeed with the exception of one experiment sulphur is definitely retained in larger amount than nitrogen.

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XLIII. THE OUTPUT OF CREATINE IN GLYCOSURIA.

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(*Received October 20th, 1915.*)

In order to obtain further evidence in support of the view that the appearance of creatine in the urine in animals, where the metabolism of carbohydrate has been interfered with, is actually due to the disturbance of the metabolic activities, and is not merely an error in observation due to the presence of aceto-acetic acid, the present experiments were carried out.

It has already been shown by Cathcart and Orr [1914] that in the case of the administration of phloridzin a creatinuria can exist without a concomitant acidosis. As the "error" in phloridzin glycosuria is generally considered to be renal, it was of some interest to examine the urine in other forms of glycosuria in which the cause is less obvious. The forms of glycosuria selected were (1) that following the injection of adrenaline, and (2) post-anaesthetic glycosuria.

PART I.

Adrenaline Glycosuria.

Since the discovery by Blum [1901] that the injection of adrenaline caused glycosuria, many confirmatory experiments have been carried out, but there is not yet perfect agreement as regards the mode of action of this substance. Noël Paton [1903] found a break-down of protein and a rise in the output of ammonia after the subcutaneous injection of adrenaline and concluded that the protein metabolism behaved as in ordinary diabetes. Underhill and Clossen [1907] were unable either to detect any increase in the catabolism

of protein or to find any alteration in the distribution of the nitrogenous constituents of the urine in adrenaline glycosuria. They believed that the action of the adrenaline was to stimulate the sugar-storing organs through the sympathetic nerves and thus prevent the storage of glycogen. The ability of the organism to utilise dextrose subcutaneously administered was not seriously impaired by the injection of adrenaline. From the fact that he was able to produce glycosuria with a dose of adrenaline which would produce a vaso-constriction in the tissues, Ritzmann [1909] concluded that the glycosuria was probably due to some sympathetic stimulation with a mechanism similar to that for vaso-constriction. On the other hand Wilenko [1912] thought that his results pointed directly to the glycosuria being due to some interference with the power of the tissues to oxidise the sugar. He found no rise in the respiratory quotient and maintained that sugar injected into an animal suffering from adrenaline glycosuria appeared in quantitative amount in the urine. Lusk [1913] however did not think that the oxidation was interfered with or that the protein metabolism was increased.

The present experiments were carried out on an Airedale bitch weighing 13·2 kilo. She was kept in an ordinary metabolism cage and fed on a fixed diet, in one experiment carbohydrate-rich and in the other carbohydrate-poor. The animal was catheterised each morning at 10 a.m. and the urine mixed with that collected in the receiver of the cage. It was diluted to a constant volume each day. The methods of analysis employed were: total nitrogen, Kjeldahl; ammonia, creatinine and creatine, Folin; urine treated in the way recommended by Graham and Poulton; urea, urease method; sugar, Benedict; and acetone and aceto-acetic acid, Messinger-Huppert. The adrenaline employed was the 1 : 1000 solution of Parke, Davis and Co.

Exp. I. Carbohydrate-rich diet: 100 g. tapioca, 70 g. oatmeal, 40 g. cane sugar, 30 g. dried skimmed milk, and 20 g. margarine.

As soon as the animal was in nitrogenous equilibrium 7 cc. of the adrenaline solution were injected subcutaneously. As a result of this injection the dog became very restless, and had free salivation but no marked dyspnoea. After the initial excitement passed off she lay down on the floor of her cage and remained quietly there for the rest of the day. She only ate a small part of her food this day. The following day, although she still looked somewhat ill, she ate all her food. On the next day at the site of injection which had become indurated a small centre of necrosis appeared. No albumin appeared in the urine either on the day of injection or any following day.

TABLE I. (*Carbohydrate diet.*)

Day	Amount in cc.	Re-action	Specific gravity	T.N. in g.	Urea N.		NH_3 N		Creatinine N.		Creatine N. in g.	Sugar in g.	Acetone and aceto-acetic acid in g.
					in g.	p. c.	in g.	p. c.	in g.	p. c.			
vi	520	acid	1011	2·85	1·55	54·3	0·143	5·0	0·126	4·4	—	—	—
vii	430	„	1013	2·88	1·52	52·7	0·157	5·5	0·121	4·2	—	—	—
viii	520	„	1011	2·82	1·77	62·8	0·122	4·3	0·129	4·6	—	—	—
ix	1100	„	1011	4·64	2·55	54·9	0·240	5·1	0·159	3·4	0·072	5·85	0·0043
x	1220	„	1008	4·22	2·09	50·0	0·253	6·0	0·177	4·2	0·045	0·61	0·0023
xi	460	„	1018	3·76	1·88	50·0	0·099 (?)	2·6	0·141	3·8	—	—	0·0035
xii	280	„	1018	3·61	1·85	51·2	0·166	4·6	0·114	3·2	0·002	—	—

It will be seen from the table, that as the result of the injection of the adrenaline there is a very definite rise in the output of total nitrogen. This rise is not due to any increase in one particular constituent but to a general increase of all the constituents estimated, as will be noted from the examination of the percentage outputs. As was to be expected, on the day of the injection there was glycosuria and associated with it the appearance of creatine. Although the output of sugar on the day following had fallen to a low level, the output of creatine was still quite marked. That this is a real creatine excretion and not merely apparent, due to the presence of aceto-acetic acid, is clear when the total acetone excreted is considered.

Exp. II. Carbohydrate-poor diet consisting of 70 g. oatmeal, 30 g. dried skimmed milk and 90 g. margarine. The same dose of adrenaline as in the last experiment was given but this time intramuscularly. The results of the injection were practically identical with those in Exp. I. Two days after the injection, in spite of the fact that the injection was into the deeper tissues, a slight necrosis of the skin appeared.

TABLE II. (*Fat diet.*)

Day	Amount in cc.	Re-action	Specific gravity	T.N. in g.	Urea N.		NH_3 N		Creatinine N.		Creatine N. in g.	Sugar in g.	Acetone and aceto-acetic acid in g.
					in g.	p. c.	in g.	p. c.	in g.	p. c.			
v	1050	amph.	1007	2·18	1·48	67·9	0·259	11·9	0·153	7·0	—	—	—
vi	1130	„	1008	2·39	1·30	54·4	0·290	12·1	0·165	6·9	—	—	0·0044
vii	1600	„	1008	3·58	1·88	52·5	0·672	18·7	0·166	4·6	0·101	0·81	0·0070
viii	1300	alk.	1007	2·60	1·21	46·5	0·852	32·8	0·151	5·8	0·023	—	0·0041
ix	1000	„	1007	2·39	1·43	59·8	0·459	19·2	0·150	6·2	—	—	0·0098
x	1000	amph.	1006	2·42	1·57	64·9	0·381	15·7	0·155	6·4	—	—	0·0059

As the result of the injection there is again quite a definite rise in the output of total nitrogen, but in this instance there is a much greater disturbance in the percentage composition. It will be noted that here there is a very marked rise in the output of ammonia and a definite rise in the excretion of total nitrogen and urea. As was to be expected the output of sugar

is much smaller although there is an increase in the output of creatine as compared with Exp. I. Here again, although there is a slight increase in the output of the total acetone, it is not sufficient to account for the creatine result.

PART II.

Post-anaesthetic Glycosuria.

It is probable that the glycosuria which follows the administration of ether is due to some disturbance of the hepatic metabolic activities. Seelig [1905], although he was unable to prove that glycosuria was a symptom of ether anaesthesia in the human subject, found that in the case of the dog a hyperglycaemia occurred after ether narcosis. As regards the glycosuria he found that it was very transitory, and even did not occur at all if, previous to the administration of the anaesthetic, an intravenous infusion of oxygen was given. He was quite certain that the glycosuria was due to the action of the ether and not to any associated symptoms arising during the narcosis.

King, Moyle and Haupt [1912] came to the conclusion that the glycosuria following ether administration was not due to asphyxia caused by the ether inhalation but was due to the intoxication with ether itself, as intravenous injection of saline saturated with ether also induced glycosuria.

As regards the influence of ether inhalation on the protein metabolism this subject has been fully discussed by Hawk [1908], who, as the result of his own experiments, found that ether anaesthesia was invariably followed by an increase in the excretion of nitrogen.

As in the previous experiments the same two diets were employed, the subject of experiment being an Airedale bitch weighing 11 kilo. On account of the transitory nature of the changes produced by ether anaesthesia, the urine of the day on which the animal was anaesthetised was divided into two parts and separately examined; (*a*) the urine which was excreted and obtained from the bladder by catheter before the anaesthetic was given, and (*b*) the urine collected for the rest of the 24-hour period after the anaesthetic.

Exp. III. Carbohydrate-rich diet. After the animal had reached a state of nitrogen equilibrium it was anaesthetised with ether for 90 minutes, from 2.30 p.m. to 4 p.m.

The animal was given a small dose (2–3 grains) of morphia before the ether was administered. Small doses of morphia have no influence on protein metabolism and have but little influence in causing glycosuria. Following the

anaesthetic there was slight vomiting but no other symptom. The animal rapidly recovered.

TABLE III. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	N. in g. T.N.	Urea	NH_3	Creatinine	Creatine	Sugar in g.	Acetone and aceto-acetic acid in g.
iv	850	acid	1006	2·25	1·43	0·238	0·148	—	—	—
v	1000	,,	1006	2·21	1·27	0·248	0·147	—	—	0·0029
vi	1030	,,	1005	2·25	1·48	0·260	0·147	—	—	0·0029
vii	220	amph.	1006	0·52	0·31	0·055	0·032	—	—	0·0005
	560	alk.	1014	1·76	1·34	0·045	0·098	0·009	0·45	0·0039
	780			2·28	1·65	0·100	0·130	0·009	0·45	0·0044
viii	800	amph.	1006	2·78	1·97	0·318	0·129	—	—	0·0054
ix	1350	acid	1005	2·29	1·31	0·507	0·138	—	—	—

There is no rise in the output of total nitrogen on the day of narcosis but there is a very slight rise in the output of the following day. There is however a slight rise in the excretion of urea and a very definite fall in the output of ammonia, a slight fall in creatinine, the appearance of a trace of creatine, a slight glycosuria and a slight increase in the output of total acetone. The appearance of the creatine was checked by the diacetyl test.

Exp. IV. A repeat experiment on the carbohydrate-rich diet but without the preliminary dose of morphia was carried out. The administration of the anaesthetic was continued for 150 minutes from 3 p.m. to 5.30 p.m.

TABLE IV. (*Carbohydrate diet.*)

Day	Amount in cc.	Reaction	Specific gravity	N. in g. T.N.	Urea	NH_3	Creatinine	Creatine	Sugar in g.	Acetone and aceto-acetic acid in g.
v	1000	amph.	1006	2·28	1·44	0·280	0·141	—	—	—
vi	1240	,,	1003	2·31	1·58	0·229	0·130	—	—	—
vii	1100	,,	1006	2·34	1·51	0·246	0·131	—	—	—
viii	420	,,	1004	0·62	0·34	0·068	0·028	—	—	0·0007
	1000	,,	1009	1·71	0·97	0·112	0·103	0·011	2·5	0·0087
	1420			2·33	1·31	0·180	0·131	0·011	2·5	0·0094
ix	1120	acid	1003	3·28	2·34	0·389	0·138	—	—	0·0076
x	1220	amph.	1005	2·39	1·50	0·314	0·123	—	—	0·0059

Again there is no rise in the output of total nitrogen on the day of the experiment but quite a marked one on the day following. The fall in the output of ammonia is quite definite although not so marked as in Exp. III. There is a slight fall in the output of urea and the excretion of creatinine is not affected. The output of creatine and sugar is much more marked, as

is also total acetone. The diacetyl test was again used to control the appearance of creatine.

Exp. V. Carbohydrate-poor diet. Without previous administration of morphia the animal was anaesthetised for 150 minutes from 2.30 p.m. to 5 p.m. Apart from slight vomiting after the anaesthetic there were no ill effects of the ether.

TABLE V. (*Fat diet.*)

Day	Amount in cc.	Reaction	Specific gravity	N. in g. T.N.	Urea	NH_3	Creatinine	Creatine	Sugar in g.	Acetone and aceto-acetic acid in g.
iv	800	acid	1011	2·64	1·65	0·179	0·116	—	—	0·0124
v	1060	„	1010	3·12	1·93	0·220	0·125	—	—	0·0113
vi	1000	„	1010	2·80	1·68	0·185	0·125	—	—	0·0097
vii	200	„	1005	0·37	0·23	0·024	0·016	—	—	0·0021
	1210	„	1007	2·54	1·65	0·214	0·106	0·007	—	0·0117
	1410			2·91	1·88	0·238	0·122	0·007	—	0·0138
viii	1000	amph.	1009	3·75	2·59	0·179	0·113	—	—	0·0135

The effect on the output of total nitrogen is again marked on the day following. In this experiment instead of a fall in the excretion of ammonia there is quite a definite rise on the day of anaesthesia. The output of creatinine is unaltered on the day of experiment but there is quite a marked fall on the day following. As regards the output of sugar none was detected. The output of creatine is a mere trace. There was a small but appreciable rise in the excretion of total acetone.

Thus in both sets of experiments, in which the modes of production of the glycosuria are very different, there is associated with the disturbance of the carbohydrate metabolism an output of creatine. There is no question in any of these experiments, with the possible exception of Exp. V, of the output of creatine being only an apparent output due to the presence of aceto-acetic acid interfering with the estimation of the preformed creatinine.

Conclusions.

1. The injection of adrenaline produces a glycosuria associated with which there is an output of creatine.

2. There is also a rise in the output of total nitrogen due to the injection of the adrenaline.

3. Ether anaesthesia is followed by a slight glycosuria and creatinuria.

4. A rise in the output of total nitrogen occurs on the day following the anaesthesia.

5. The output of creatine in these experiments is not "apparent," due to the presence of aceto-acetic acid, but is a real output.

These experiments were carried out at the suggestion and under the direction of Dr E. P. Cathcart, to whom I offer my thanks. Dr Cathcart is responsible for the injections.

The expenses were in part defrayed by a grant made to Dr Cathcart by the Carnegie Trust.

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XLIV. ACIDOSIS AND SOME OF THE FACTORS WHICH INFLUENCE IT.

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(*Received October 20th, 1915.*)

Introduction.

The excretion of acetone, aceto-acetic acid, and β-hydroxybutyric acid, to which the name of acidosis was applied by Naunyn, is of great interest to the physiologist, the pathologist and the clinician. Its importance lies in the fact that the output of these substances affords us practically the only clue to the faulty metabolism of fat. It is true that a certain amount of information regarding the quantitative metabolism of fat has been acquired by a study of the respiratory quotient, but by this method we gain little information concerning the qualitative or intermediary metabolism. All that has been ascertained with certainty is that fat is, under normal circumstances, completely oxidised to carbon dioxide and water.

It has long been known that acetone is a normal constituent of the body fluids. It can be detected in small quantities in the blood, in the saliva, in the breath, in the liquor amnii and in the urine. Of these channels of excretion, that by way of the urine is of the most importance and the estimation of the urinary acetone has been adopted here. The other acetone bodies, namely aceto-acetic acid and β-hydroxybutyric acid, are also mainly found in the urine and some account of them will be given in our experiments.

One of the great questions which has been long under discussion [*vide* Magnus Levy, 1908; v. Noorden, 1907; Geelmuyden, 1900; Forssner, 1909, etc.] is the source of the acetone bodies, some workers maintaining that they were derived from fats alone, others just as actively supporting the contention that proteins played a part in their production. Modern work has shown,

and the present communication will bear out the conclusion, that fats may be considered the main source, though, of course, it must be remembered that Dakin [1912] and others have clearly demonstrated that certain constituent amino-acids of the protein molecule can, on decomposition, yield aceto-acetic acid.

Methods.

The total nitrogen was estimated by Kjeldahl's method and the ammonia by Folin's method. The preformed acetone was estimated by Folin's method, using an apparatus modified to suit the occasion. The total acetone (acetone + aceto-acetic acid) and β-hydroxybutyric acid were estimated by Shaffer's [1908] method. Several workers have investigated this method of Shaffer's and all agree that when estimating the amount of acid in the sodium salt 90–96 % of the theoretical value is found. Kennaway [1914] also finds a similar value but is not satisfied that such a small error is present when the method is applied to urine. He states that "it is of course much more difficult to find out whether the acetone obtained from urine by oxidation is derived wholly from β-hydroxybutyric acid and there seems no satisfactory way of doing this." Certain variations in the results obtained by this method in previous experiments aroused some suspicions as to the possibility of a fallacy, so that before the present experiments were proceeded with, a series of control analyses was made. It was found for instance, when estimating total acetone in a normal urine of a subject on constant normal diet, that the amount of acetone varied greatly from day to day, and, as different quantities of basic lead acetate solution were added to each urine, according to the variations in the quantity of urine, the influence of this salt was naturally investigated first. Accordingly, dry basic lead acetate was heated in a test tube which was connected with another tube containing a measured volume of an alkaline solution of N/10 iodine. After heating 2 g. of the salt for a couple of minutes and then titrating the iodine solution in the ordinary way it was found that some substance capable of combining with iodine to the extent of 3·6 cc. N/10 iodine had been distilled off. In another experiment 10 cc. basic lead acetate solution (U.S.P.) were put into a test tube, 1 cc. H_2SO_4 added, as in estimation of acetone ($PbSO_4$ was precipitated), and the tube connected up as before with another test tube containing a measured volume of N/10 iodine in alkaline solution. The lead acetate tube was boiled for about a minute and some substance was given off which caused a loss of 0·8 cc. N/10 iodine. In view of the fact

that one of the ordinary laboratory methods for the preparation of acetone is the dry distillation of calcium acetate, it may be assumed that in the present instance the iodine-combining substance distilled off was acetone. This assumption was confirmed by a positive result with the nitroprusside test.

Several other blank determinations were then carried out by the ordinary Shaffer method, but using water in place of urine.

40 cc. basic lead acetate solution (U.S.P.) were added to 250 cc. of water and the whole well shaken. 20 cc. ammonium hydrate were added, mixed, made up to 500 cc. and again well shaken. After standing for some minutes the liquid was filtered. 200 cc. of the filtrate were made up with water to 600 cc. and 15 cc. pure sulphuric acid added. A second 200 cc. were treated the same way. These were then distilled, using an ordinary condenser, until 200 cc. had collected in each receiving flask. The distillate was re-distilled, boiling for twenty minutes with 10 cc. 10 % sodium hydrate. Previous to each distillation the receiving flasks contained 300 cc. of cold water, and care was taken that the connecting tube dipped beneath the surface and that the water did not become hot. The acetone in the distillate was estimated by the iodine titration method. As the figures given show, a small quantity of acetone was evolved in each case.

N/50 iodine cc.	N/50 thiosulphate cc.	Difference cc.
5	3·2	1·8
5	3·3	1·7
5	3·05	1·95
5	3·35	1·65
4	2·25	1·75
4	2·4	1·6
2	0·5	1·5
2	0·7	1·3
4	2·2	1·8
4	2·05	1·95

The difference is seen to be fairly constant and to have an average of 1·7 cc. N/50 iodine.

When carrying out estimations in diabetic urine, or in urine containing a large quantity of acetone bodies, this error may be relatively small, but in normal urine a considerable error is always produced, in some cases more than 100 %.

When analysing normal urines—i.e. practically sugar free—10 cc. of the lead acetate solution were used if less than 100 cc. of urine were taken, 20 cc. solution for 100–150 cc. of urine and 40 cc. solution if more than 150 cc. of

urine. Accordingly after the use of 40 cc. solution, 1·7 cc. was subtracted from the iodine difference obtained, and after 20 cc. or 10 cc. one-half or one-quarter of this amount.

The method, thus corrected, was found to be an excellent one for estimating even very small quantities of acetone.

In the estimation of β-hydroxybutyric acid Shaffer himself admits that there is an error of about 10 %.

Several check experiments were carried out, but, as the results obtained were so discordant, and as the other methods for the estimation of this substance were too slow for our purpose, β-hydroxybutyric acid was estimated in only a few of the experiments recorded in the present paper.

Excretion of Acetone by the Normal Individual[1].

A comparatively large number of investigations have been carried out from time to time on the normal urinary acetone output. The results which have been obtained vary to a certain extent, the differences being in part due to the differences in the method of estimation. The results which workers like Hirschfeld [1895] and Geelmuyden [1900] have obtained show that during the twenty-four hours 10–30 milligrams of total acetone are excreted in the urine. The results which I have found in the case of several healthy individuals are in complete agreement with these. It was found also, that on a constant diet the total acetone excreted is practically constant in amount. The quantity found is influenced by the diet, being greater with a high protein intake than with a low one. In one experiment of four days duration, where 400 g. of protein (mainly consisting of caseinogen) per diem were taken, 53 mg. of acetone + aceto-acetic acid were found in the fourth day's urine. The total nitrogen in the same urine was 52·16 g., and in the faeces 2·56 g.

In another experiment where a constant "standard" diet was taken, containing 118 g. of protein, derived from milk, eggs and bread, the amount of total acetone excreted daily in the urine lay between 15·7 mg. and 18·2 mg. The lowest recorded output of acetone in my own case was 5·2 mg. found on the third day on a diet of 100 g. cane sugar per diem.

While carrying out some investigations on post-anaesthetic diaceturia some very low total acetone outputs were found. One patient, who had been operated on for appendicitis, under chloroform and ether, excreted only

[1] The subject employed in all the experiments detailed in this paper was the writer himself.

29·7 mg. total acetone during the four days following the anaesthetic, while another patient, who exhibited no post-anaesthetic diaceturia, excreted 5·1 mg. on the fourth day after a general anaesthetic.

Another point of some interest arises, namely whether, as in the case of the nitrogenous products of metabolism, there is a diurnal variation in the excretion of these acetone bodies. In order to elucidate this point the following experiment was carried out. The standard diet detailed below was taken for five consecutive days. On the first day, the food was taken in three equal parts, at intervals of eight hours. On the second day, two-thirds of the food was consumed in equal amounts two hourly from 7 a.m. till 11 p.m. At 11 p.m. the remaining third of the food was taken. On the third, fourth and fifth days one-twelfth of the food was taken two hourly, day and night. The urine was also collected two hourly and the two hourly output of total acetone and also of total nitrogen for the third, fourth and fifth days are given in the following table (Table I).

Diet.

Wheaten bread	360 g.	Protein	107 g.
Dried separated milk ...	252 „	Carbohydrate	480 „
Walnut butter	72 „	Fat	73 „
Cane sugar	211·2 „	Energy value	3081 cal.

It is seen that the rate of excretion of the acetone bodies is not absolutely constant from hour to hour, but that any variation detected cannot be related to any particular period of the twenty-four hours. Forssner [1909] obtained a very similar result.

Influence of the Nature of the Diet.

Another factor of very considerable importance is the actual nature of the diet. It has long been recognised that, when the external supply of carbohydrate is greatly diminished or entirely cut off, as in starvation, the condition known as acidosis rapidly ensues. The less carbohydrate there is metabolised, relative to the fat metabolised, the greater will be the degree of acidosis and the more acute its onset.

In the following experiments, the influence of fat and protein on the output of preformed acetone and of total acetone on a standard diet was investigated. The standard diet (see previous experiment) was taken for four days. The food was taken every four hours throughout that time. The urine was collected four hourly during the last three days. At 8 a.m. on the third day 300 g. of pure olive oil were taken in addition to the other

TABLE I.

Day of diet	Hour	Total acetone mg.	Total nitrogen g.
3	7–9 a.m.	1·76	1·43
	9–11 „	1·68	1·43
	11–1 p.m.	1·35	1·25
	1–3 „	1·66	1·34
	3–5 „	1·82	1·29
	5–7 „	1·95	1·26
	7–9 „	1·73	1·20
	9–11 „	2·53	1·14
	11–1 a.m.	1·95	1·23
	1–3 „	1·98	1·32
	3–5 „	2·18	1·31
	5–7 „	1·40	1·51
4	7–9 „	1·37	1·62
	9–11 „	1·64	1·62
	11–1 p.m.	1·37	1·34
	1–3 „	1·85	1·41
	3–5 „	1·73	1·13
	5–7 „	1·98	1·50
	7–9 „	1·56	1·39
	9–11 „	1·58	1·43
	11–1 a.m.	1·72	1·28
	1–3 „	1·45	1·52
	3–5 „	1·79	1·55
	5–7 „	1·66	1·58
5	7–9 „	1·63	1·51
	9–11 „	1·81	1·65
	11–1 p.m.	1·43	1·63
	1–3 „	1·24	1·53
	3–5 „	1·31	1·57
	5–7 „	1·24	1·49
	7–9 „	1·47	1·39
	9–11 „	1·40	1·44
	11–1 a.m.	1·19	1·35
	1–3 „	1·23	1·29
	3–5 „	1·16	1·64
	5–7 „	1·63	1·79

food. The oil was well emulsified with water containing 3 g. anhydrous potassium carbonate. The urine collected at midnight and at 4 a.m. was examined in the morning and the remainder examined as collected.

The experimental data given below (Table II) show that the catabolism of fat in the presence of an abundance of carbohydrate does not produce any increase in the output of the acetone bodies in the urine, but rather a slight temporary decrease. This is probably due to the protein-sparing action of the fat, as indicated by the fall in the total nitrogen. As protein is responsible for some of the acetone present, it might be assumed that

a diminished protein metabolism would result in less total acetone being formed. It will be noted that the decrease in the output of the acetone bodies was most marked in the case of the preformed acetone.

TABLE II.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
2	4–8 a.m.	2·15	1·63	75·6	2·59
	8–12 noon	2·88	1·63	56·4	2·90
	12–4 p.m.	2·64	1·97	74·8	2·55
	4–8 „	2·25	0·81	36·1	2·59
	8–12 m.n.	2·78	—	—	2·76
	12–4 a.m.	2·39	1·16	48·5	2·58
3	4–8 „	2·39	0·99	41·3	2·60
	8–12 noon*	2·44	0·99	40·4	2·70
	12–4 p.m.	1·93	1·39	72·0	2·52
	4–8 „	2·20	0·28	12·7	2·16
	8–12 m.n.	1·96	0·52	26·6	1·88
	12–4 a.m.	2·30	0·87	37·8	2·32
4	4–8 „	2·35	0·52	22·3	2·43
	8–12 noon	2·30	0·58	25·2	2·65
	12–4 p.m.	1·79	0·46	26·5	2·35

* 300 g. olive oil at 8 a.m.

When protein is added to the standard diet in place of the oil a definite increase of the total acetone results (Table III).

TABLE III.

Day	Hour	Total acetone mg.	Total nitrogen g.
3	7–11 a.m.	3·72	2·56
	11–3 p.m	3·48	2·37
	3–7 „	3·60	2·32
	7–11 „	3·99	2·32
	11–3 a.m.	3·14	2·16
	3–7 „	4·08	2·25
4	7–11* „	3·96	3·54
	11–3 p.m.	4·93	4·65
	3–7 „	4·62	3·88
	7–11 „	3·29	3·34
	11–3 a.m.	3·75	3·22
	3–7 „	4·20	3·08

* 100 g. caseinogen at 7 a.m.

The standard diet was taken for four days, during the first two days eight hourly, and during the next two days four hourly, the food being equally partitioned as in previous experiments. At 7 a.m. on the fourth day 100 g. of caseinogen were taken along with the usual food. A sample of the caseinogen boiled with dilute hydrochloric acid and filtered did not reduce Fehling's solution.

Unfortunately, on this occasion the preformed acetone was not estimated, and it is therefore impossible to say which of the acetone bodies increased, or whether both were raised in amount. The figures are in keeping with those of other experiments, and all indicate that the greater the protein break-down, the higher is the amount of total acetone excreted.

Effect of Preceding Diet on the Acidosis of Starvation.

The next series of experiments was carried out chiefly to observe the effect of the preceding diet on the acidosis of starvation. This acidosis is almost certainly due to the great deficiency in the supply of carbohydrate available for immediate use. The sudden increase in the urinary acetone bodies probably occurs when the stored carbohydrate has all been utilised. Almost every experiment points to the fact that the animal organism prefers to derive its energy from carbohydrate rather than from fat, or even from protein. Carbohydrate is the most easily oxidised food-stuff and is a better protein sparer than fat, and for these reasons, when the body is starving and requiring the maximum amount of energy with the minimum of protein destruction, carbohydrate will be used so long as it is available. When this has been exhausted, most of the energy would appear to be derived from fat. The ratio of the fat oxidised to the carbohydrate oxidised then becomes much higher than it was previously, and, when this ratio rises above a certain definite value, abnormal quantities of acetone bodies appear in the urine. The results of experiments done on this subject point to the existence of a quantitative relationship, which has a very definite minimal value, between the fat and the carbohydrate utilised to prevent acidosis. In the light of these observations the initial rise in the excreted acetone bodies is readily understood.

Starvation following a high Carbohydrate Diet.

Second day before the fast—normal diet to which was added cane sugar and other carbohydrate-rich food.

Pre-day's diet consisted of:

Cane sugar	...	450 g.	Cornflour	...	40 g.
Tapioca	...	115 g.	Milk ...	...	50 g.
Bread ...	...	300 g.	Butter ...	...	15 g.
Potatoes	...	150 g.	Lean meat	...	50 g.

Some jam.

This gives a carbohydrate intake of some 757 g. The urine was collected from the time at which the last meal was taken. None of the urine collected reduced Fehling's solution, though that on the pre-day did so slightly. The period of fasting was three days. The results are shown in Table IV.

TABLE IV.

Day of fast	Total acetone mg.		Total nitrogen g.
1	20·51		4·17
2	28·79		4·41
3	138·96	First 12 hrs 30·22 Second „ 108·74	—

Starvation after high Protein Diet.

Second day before—high protein diet

Pre-day: Caseinogen ... 460 g. Bread ... 150 g.
Lean meat ... 100 g. Butter ... q.s.
2 eggs.

The last meal consisted of 120 g. caseinogen and the urine was collected from that time. The period of fast was three days (see Table V).

TABLE V.

Day of fast	Total acetone mg.		Total nitrogen g.
1	26·75		34·72
2	77·84		15·47
3	235·85	First 12 hrs 57·58 Second „ 178·27	18·92

Starvation after high Fat Diet.

Pre-day: 300 g. pure olive oil well emulsified with water and 3 g. K_2CO_3.
1 egg and 30 g. oatmeal at 10 a.m.
90 g. lean meat at 1 p.m.

Last meal, consisting of 150 g. olive oil, at 6 p.m. and the urine collected from that time. The period of fast was two days (see Table VI).

TABLE VI.

Day of fast	Total acetone mg		Total nitrogen g.
1	61·70		7·21
2	259·01	First 12 hrs 98·30 Second „ 160·71	10·53

These results show conclusively that the acetone output in the early days of starvation *is* governed by the nature of the preceding diet.

In the first experiment there was sufficient stored carbohydrate to prevent acidosis for some forty-eight hours. The output of total acetone during that period was not above the normal. During the third day, however, a marked increase of the bodies in question occurred, 30·2 mg. being excreted in the first twelve hours and 108·7 mg. in the second. It will be noticed that the onset of acidosis is very rapid. This is found to be the case in almost every experiment, and, as will be shown later, its disappearance is even more rapid. This rise in the total acetone may occur at any part of the twenty-four hours, neither day nor night having any influence over it.

The second experiment shows that a large intake of protein previous to starvation also delays the onset of acidosis. The delay is not due to the presence of protein itself, for this is rapidly deaminised after absorption, the nitrogen-free part of the molecule being catabolised or stored, probably as carbohydrate. In all probability it is the carbohydrate derived from protein which postpones acidosis, and, as there would be less carbohydrate stored up at the beginning of this fast than at the beginning of the previous one, acidosis would here be apparent at an earlier period. On the first day's starvation 26·75 mg. of total acetone were found in the urine; but as the total nitrogen was 34·72 g. a considerable part of this acetone was almost certainly derived from the protein. On the second day the total acetone found was relatively much greater than the normal. On the third day, especially in its latter half, a further and more pronounced increase took place.

As one would expect, when a high fat diet precedes starvation, there is an increased output of acetone bodies almost from the beginning, due to the fact that this fat during its combustion would draw on the stored carbohydrate. The sudden rise in the total acetone excreted is here observed on the second day.

Acidosis in Starvation.

The amount of acetone excreted in the urine during starvation has been determined by Brugsch [1905], Bönniger and Mohr [1906] and others. On the first day the quantity is generally small. On the second day variable amounts are found, but by the third day a marked rise is always observed and the increase continues till about the fifth day. A diminution in the amount of the acetone bodies then occurs, and a more or less constant output of 0·4–0·8 g. is recorded, this being about half or less of the quantity found when the output is at its maximum.

Brugsch for example found the following results for the twenty-third to the thirtieth day of a thirty days fast by Succi.

Day	Total acetone mg.	Total nitrogen g.
23	569	5·837
24	410	6·410
25	463	6·272
26	569	6·182
27	525	6·302
28	339	4·437
29	242	4·193
30	115*	8·421

* Lemonade with sugar taken.

To what is this fall in the total acetone output due? Acidosis can be diminished by the catabolism of carbohydrate or of protein, and if only for economic reasons, the former is the more likely cause of the change, unless some altogether unknown factor in the animal metabolism plays a part. We have next to find the source of this carbohydrate, assuming that it has brought about the decreased acetone excretion.

Landergren [1903] stated that the sole source of carbohydrate in starvation was protein. Now there is less protein metabolised per diem in the second week of starvation than in a like period during the first few days, and consequently at the later period less carbohydrate would be produced, leading to an increased fat : carbohydrate ratio. This would inevitably be accompanied by an accentuated acidosis. On the contrary, however, the reverse is found, so that another cause must be sought.

Although the conversion of fat or fatty acids into carbohydrate has not been proved, the possibility of such a change has been put forward by several physiologists of repute, and a certain amount of evidence in favour of it has accumulated. It does seem possible that, under special circumstances, when, for example, the body is in great need of carbohydrate, such a change may occur. That sugar can be derived from glycerol is already known, but whether this occurs in the body remains to be proved. A certain amount of evidence in favour of the view that in diabetes an increased output of sugar follows the administration of glycerol does certainly exist. If fat yields carbohydrate the diminished acidosis might readily be explained.

Factors influencing Acidosis.

The results which have been dealt with up to the present deal with the factors which influence the onset of acidosis, and it is now proposed to consider some of the factors which influence the acidosis after it is definitely present.

1. *Effect of feeding Carbohydrate.*

The subject had been on a fat diet (320 g. pure olive oil per diem) for three days. At 7 a.m. on the fourth day 100 g. of cane sugar were taken and the same again at 2 p.m. At 6.30 p.m. and 11 p.m. eggs and bread and butter were taken.

Table VII gives the results for the third (the last) day of the oil diet and for the fourth day when carbohydrate was given.

TABLE VII.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
3	7–10 a.m.	89·83	11·84	13·1	1·94
	10–1 p.m.	85·72	11·60	13·5	1·70
	1–4 „	91·53	10·73	11·7	1·53
	4–7 „	88·87	11·84	13·3	1·49
	7–10 „	104·10	13·23	12·7	1·52
	10–1 a.m.	91·28	16·59	18·2	1·30
	1–4 „	114·49	18·22	15·9	1·76
	4–7 „	141·81	22·34	15·7	1·67
4	7–10* „	50·43	14·16	28·1	1·29
	10–1 p.m.	12·23	6·27	51·0	1·24
	1–4* „	9·96	5·57	55·9	1·19
	4–7† „	4·50	—	—	0·97
	7–10 „	2·27	1·86	81·8	1·21
	10–7† a.m.	4·98	3·48	69·9	3·60

Day	β-Hydroxybutyric acid
3	1273 mg.
4	206 „

* 100 g. of cane sugar at 7 a.m. and 2 p.m.

† Eggs, bread and butter at 6.30 p.m. and 11 p.m.

2. *Effect of feeding Protein.*

The subject had been fasting for two days, the fast being preceded by a high fat diet. At 6 p.m. on the second day 100 g. of caseinogen were taken, and no more food for twelve hours afterwards (see Table VIII).

TABLE VIII.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
2	6–10 a.m.	50·19	7·35	14·64	1·88
	10–2 p.m.	53·33	7·54	14·15	2·20
	2–6* „	57·20	8·12	14·21	1·83
3	6–8 „	15·38	3·87	25·15	1·74
	8–10 „	6·91	3·19	46·15	1·42
	10–2 a.m.	10·54	2·71	25·68	4·28
	2–6 „	15·38	2·71	17·61	3·46

* 100 g. caseinogen at 6 p.m.

As will be seen from the preceding tables both carbohydrate and protein cause a rapid diminution in the amount of acetone bodies found in the urine.

(1) The sudden disappearance of acidosis after feeding sugar is very striking, the output of total acetone dropping from 141·8 mg. in one period of three hours to 50·4 mg. in the subsequent three hours. This change actually occurred even more rapidly than the figures indicate, since some thirty minutes would elapse before the sugar was available in the body, and during this time acetone bodies would be excreted at the same rate as before the sugar was taken. Evidently the presence of carbohydrate causes a profound alteration in the course of fat catabolism. The suddenness of the change suggests that it is the latest stages in the oxidation of the fatty acids which are affected. It is not improbable that the first stages in the oxidation of fat are accomplished independently of carbohydrate, but that the later stages of the catabolism are diverted from the normal in the absence of this material. At least the glyceryl radicle of fat can be oxidised in absence of carbohydrate.

The diminished acetone output was accompanied by an immediate fall in the total nitrogen, so that presumably the same moiety of carbohydrate which is available for the prevention of acidosis acts also as a protein sparer.

(2) As both absorption and combustion of protein take place more slowly than they do in the case of carbohydrate, the effect of protein on the metabolism is longer in becoming apparent. The acidosis was quickly checked, but not reduced to normal, probably because the caseinogen did not yield sufficient carbohydrate or did not yield it quickly enough.

The marked rise in the total nitrogen (about 100 %) in the first two hours after feeding showed that deamination was already going on.

3. *Effect of feeding Fat.*

The subject had been fasting for three days. 200 g. pure olive oil, well emulsified, were taken immediately at the close of the fasting period. No further food was taken for twelve hours.

The effect of fat will be seen from the following table (Table IX).

TABLE IX.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
3	8–12 noon	27·68	3·68	13·28	1·89
	12–4 p.m.	38·12	4·64	12·18	2·02
	4–8 „	42·95	4·64	10·74	1·74
4	8–10* „	10·31	3·09	29·97	0·90
	10–12 m.n.	13·20	3·35	25·38	0·74
	12–8 a.m	91·81	19·34	21·07	—

* 200 g. olive oil taken at 8 p.m.

The figures show that after feeding fat, there was an immediate drop in the total acetone output during the first four hours and perhaps longer. This was succeeded by a very definite rise. A change was apparent during the first two hours so that the effect of the oil on the metabolism must soon have been felt. It must be remembered that the oil, being well emulsified, would be quickly absorbed.

More than one explanation is suggested for this unexpected decrease. The fat supplied certain energy needs which in the starving organism were probably covered by carbohydrate thus liberating carbohydrate for metabolic purposes. Again the oil might be more readily utilised than the body fat and consequently a sparing of the latter would occur, while the former would be broken down. If the first stage of this oxidation do not require the presence of carbohydrate in order to be normally carried out—and this is probably the case—then there will be a relatively greater amount of carbohydrate available to bring about the complete oxidation of the already partially broken down body fat, carbon dioxide and water being excreted in place of acetone bodies.

This decrease in the degree of acidosis, following immediately on taking fat, seems to be an argument in favour of the first stages in the oxidation of fat occurring without the aid of carbohydrate. A similar result will be alluded to in a future experiment. The subsequent rise in the total acetone output is again due to the disturbed quantitative relationship between the carbohydrate and the fat catabolised.

4. *Effect of feeding Glycerol.*

In another experiment 100 g. of glycerol were fed at 8 p.m., after fasting for three days. The results obtained are given in Table X.

TABLE X.

Day	Hour	Total acetone mg.	Total nitrogen g.
3	8–12 noon	31·23	4·00
	12–4 p.m.	60·10	2·95
	4–8 „	86·93	1·88
4	8–10* „	37·86	1·15
	10–12 m.n.	12·47	0·90
	12–8 a.m.	5·42	5·04

* 100 g. glycerol were taken at 8 p.m.

Glycerol also causes a disappearance of acidosis. During the first two hours the decrease in the total acetone was slight, but by the end of four hours a marked diminution was observed. A normal amount of total acetone was excreted between the fifth and twelfth hours inclusive after taking glycerol.

How does glycerol act? It may possibly be converted into sugar. The greatly diminished acidosis which followed its ingestion suggests that such a conversion has taken place. This change may not occur under normal circumstances, but when carbohydrate is scarce such a conversion may be postulated. Again glycerol, as an energy yielding substance, will cause the body fat to be spared, thereby reducing the fat : carbohydrate ratio and bringing about a diminished acidosis. Such a marked change as was recorded could not have occurred, however, had glycerol acted only in this way.

5. *Effect of feeding Alcohol.*

The pre-day diet contained approximately 80 g. protein, 400 g. carbohydrate and 80 g. fat.

On each of the two following days 40 g. of absolute alcohol were taken and no other food. The alcohol was well diluted with water and taken at regular intervals from morning till night. Table XI shows the results obtained.

TABLE XI.

Day	Total acetone mg.	Total nitrogen g.	NH_3 g.
1	132·2	11·49	0·416
2	192·0	14·83	0·588

The results confirm Hirschfeld's [1895, 1897] statement that alcohol does not prevent acidosis.

Being a source of energy it will probably cause a slight diminution in the total acetone by sparing fat, but it exerts no such influence as does carbohydrate, protein or glycerol on the later stages of the oxidation of fat.

Influence of high Fat Diet on Acidosis.

A series of experiments was next carried out to find the effect of protein-free fat-rich diets on the urinary output of acetone bodies. The results of two of these experiments are compared with results obtained in experiments where the fat was omitted from the diet and carbohydrate alone fed. Unfortunately, owing to the disagreeable nature of the diet and to the discomfort it produced, the experiments could not be carried out for longer periods than three days. At the beginning of each period, during which oil was being taken, 2–3 g. calcium carbonate were taken. Diarrhoea was altogether absent and, in fact, the bowels rarely moved. This is in marked contrast with the extremely severe diarrhoea met with on a fat diet, when butter is the fat ingested.

Pure Fat Diet.

The pre-day diet contained very little carbohydrate and consisted of 6 eggs, 320 g. filleted cod, 280 g. fresh haddock, 170 g. potatoes, 50 g. bread, butter, q.s.

Total carbohydrate about 84 g., the last of which was taken seventeen hours before the oil diet began.

Diet days. 80 g. pure olive oil, emulsified as before, using 1·5 g. potassium carbonate, were taken four times a day at equal intervals, viz. 7 a.m., 1 p.m., 7 p.m., 1 a.m. This was continued for three days. The urine was collected three hourly from 7 a.m. on the first day. The first table (Table XII) is a daily chart, the second (Table XII A) a three hourly one.

TABLE XII.

Day	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	147·9	37·4	25·3	411	14·78
2	389·2	61·7	15·8	616	14·96
3	817·6	116·4	14·3	1273	13·50
Day after	84·4	32·1	38·1	206	9·51

TABLE XII A.

Day	Hour	Total acetone mg.	Preformed acetone mg.	Preformed acetone %	Total nitrogen g.
1	7–10 a.m.	2·25	—	—	2·57
	10–1 p.m.	4·01	0·81	20·2	1·94
	1–4 „	8·66	2·21	25·5	1·83
	4–7 „	22·35	6·50	29·0	1·43
	7–10 „	11·72	4·13	35·3	1·31
	10–1 a.m.	35·90	8·82	24·6	1·27
	1–4 „	35·32	8·12	22·9	1·88
	4–7 „	27·71	6·85	24·7	2·00
2	7–10 „	53·33	7·25	13·6	1·92
	10–1 p.m.	49·70	8·47	17·0	2·05
	1–4 „	52·85	7·89	15·0	1·82
	4–7 „	48·98	6·61	13·5	1·45
	7–10 „	41·73	6·15	14·9	1·32
	10–1 a.m.	34·72	8·47	24·4	1·59
	1–4 „	42·94	8·34	19·4	1·66
	4–7 „	64·94	8·53	13·1	1·87
3	7–10 „	89·83	11·84	13·1	1·94
	10–1 p.m.	85·72	11·60	13·5	1·70
	1–4 „	91·53	10·73	11·7	1·50
	4–7 „	88·87	11·84	13·3	1·49
	7–10 „	104·10	13·23	12·7	1·52
	10–1 a.m.	91·29	16·59	18·2	1·30
	1–4 „	114·49	18·22	15·9	1·76
	4–7 „	141·81	22·34	15·7	1·67

A gradual increase in the urinary acetone bodies is observed from beginning to end. It will be noticed, however, during the second and third days of the diet, that on most occasions there was less total acetone in the urine passed during the second three hours after taking oil, than during the first three hours. A similar occurrence was observed and commented on in a previous experiment where oil was fed during starvation.

How long the increasing acidosis would have continued it is difficult to say. Would it cease earlier or later when fat is being fed than when the body is living on its own fat? Would the acidosis become so great as to produce serious symptoms? These questions must remain unanswered till a more prolonged series of experiments is carried out. Is there a more pronounced acidosis when the body is living on food fat than when living on its own fat? Forssner [1910] found that the addition of fat to a diet of fat and protein caused a rise in the total acetone excreted. In the experiment at present under consideration there is a much greater acetone output than in any of the starvation experiments. Moreover the highest acetone output during starvation was found after a high fat diet, when the body was using

external fat as well as its own fat, so that as far as these results go this question may be answered in the affirmative. Forssner gave as a reason for this either that food fat is broken down in a different manner from body fat or that part of the former is converted into acetone bodies after ingestion.

The preformed Acetone.

The figures in this experiment, as well as those of other experiments communicated in this paper, show that the greater part of the total acetone is present in the form of aceto-acetic acid. Where the amount of total acetone is small the percentage of preformed acetone is high, perhaps 50–60 or more, but where the total acetone output is high only about 15 % of it is in the form of preformed acetone. A similar result was obtained by Piper [1913] and led him to use the term diaceturia in preference to acetonuria.

It is not to be supposed that the small quantity of acetone found in the urine is all that is produced in the body. This substance is very volatile and is excreted in larger quantities in the breath than in the urine. In order to test this point the following experiments were carried out.

On the evening of the third day on this diet about half of the air expired during twenty minutes was passed through a measured quantity of alkaline N/50 iodine with the result that 1·5 mg. of acetone were detected. Owing to the lack of a proper respiratory apparatus it was impossible to carry out an absolute quantitative examination of the expired air, and the result obtained must be regarded as representing a mere fraction of the acetone present. The breath had a marked odour of acetone.

Effect of adding Carbohydrate to a Fat Diet.

The addition of sugar to the diet causes a great reduction in the amount of acetone bodies excreted. The greater the weight of sugar added the less is the amount of acetone bodies found.

In order that the results might be uniform the quantities of protein and fat taken on the day preceding each of this series of diets were practically the same in every case, the former amounting to some 180 g. and the latter to about 80 g. The same quantity of carbohydrate was taken on each pre-day as was taken on the succeeding diet days. The olive oil was always emulsified as already described.

The addition of 60 g. cane sugar reduced the daily amount of urinary acetone from decigrams to centigrams, but the well-marked increase in the total acetone from day to day showed that a greater amount of sugar was

required to prevent acidosis. With the addition of 100 g. of cane sugar there is still more acetone than normal after the first day. When 150 g. of sugar were added to the fat more acetone was excreted on the third day than on the first, but the increase was slight. The amount of cane sugar required to prevent acidosis when 320 g. of fat are fed is then about 150 g. If all the oil is utilised the fat : carbohydrate ratio will be a little more than 2 : 1.

TABLE XIII.

Diet: 60 g. cane sugar—320 g. olive oil.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	40·13	256	10·97
2	60·56	210	9·18
3	81·11	499	7·55

TABLE XIV.

Diet: 100 g. cane sugar—320 g. olive oil.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	15·37	173	11·54
2	35·01	268	10·85
3	69·72	234	4·83

TABLE XV.

Diet: 150 g. cane sugar—320 g. olive oil.

Day	Total acetone mg.	Total nitrogen g.
1	10·12	6·94
2	21·47	4·73
3	24·05	2·24*

* Repeated analysis showed this result to be correct.

Effect of feeding Carbohydrate alone.

When 30 g. of sugar per diem are taken the amount of acetone found is only one-sixth to one-eighth of that found when the organism is totally deprived of food (Table XVI).

TABLE XVI.

Diet: 30 g. cane sugar per diem.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.	NH_3 g.
1	27·86	174	9·12	0·366
2	25·47	254	10·35	0·337
3	34·91	601	10·34	0·396

With 60 g. sugar more acetone was found than in the previous experiment. This must be due to the effect of the preceding diet, though the pre-day diet was almost the same in each case (Table XVII).

TABLE XVII.

Diet: 60 g. cane sugar per diem.

Day	Total acetone mg.	β-Hydroxybutyric acid mg.	Total nitrogen g.
1	21·64	186	8·51
2	45·51	224	8·01
3	79·05	214	9·70

TABLE XVIII.

Diet: 100 g. cane sugar per diem.

Day	Total acetone mg.	Total nitrogen g.
1	6·6	6·94
2	9·7	11·28
3	5·2	9·81

When 100 g. of cane sugar are taken (Table XVIII) in the course of the twenty-four hours, there is less total acetone found in the urine than is found when one is taking a normal diet. This quantity of sugar is sufficient then to prevent acidosis when no fat is taken in the food. As there was not much work done, about 2500 cal. would supply sufficient energy for one day. The sources of energy would be roughly as follows:

100 g. cane sugar	380 cal.
9 g. N = 57 g. protein	235 cal.
	615 cal.

The remaining 1885 cal. would be mainly derived from 203 g. body fat. Here again the fat : carbohydrate ratio is a little more than 2 : 1. While it is freely admitted that these two experiments giving a similar fat : carbohydrate ratio are not absolutely conclusive, the assumption may be made that there is a definite minimum fat : carbohydrate ratio required to prevent acidosis, and that this ratio is a little more than 2 : 1.

Effect of the various Diets on the Total Nitrogen.

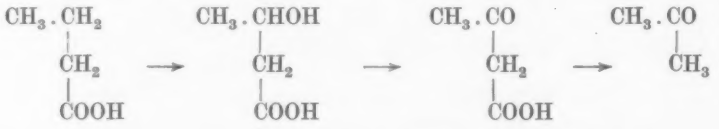
It was also noticed that the more sugar there was added to the diet the less was the amount of total nitrogen found in the urine, and that on the diets containing fat + carbohydrate less total nitrogen was eliminated than on the diets of carbohydrate alone. In each experiment where the diet consisted of olive oil a feeling of intense lassitude and dullness was present,

accompanied by abdominal discomfort. The former symptom was never absent, while the latter persisted for about two hours after taking oil. No vomiting occurred in any of the experiments recorded although a feeling of intense nausea was frequently present. A few experiments had to be discontinued owing to rejection of the oil even when taken through a stomach tube. These symptoms were less marked when sugar was added to the diet. If carbohydrate (100 g. cane sugar) was taken at the conclusion of the oil period all feelings of discomfort disappeared in less than two hours, although the limbs remained "tired" for some five hours longer. During these diets thirst was rarely experienced and little water was taken. Immediately after taking food an intense thirst developed and remained for about a day.

The significance of the Acetone Bodies.

It is known that in the body the fatty acids are oxidised in the β-position, and that eventually butyric acid is always formed [Ringer, 1913].

The prevailing opinion is that the oxidation of this acid in the β-position gives rise to β-hydroxybutyric acid as a normal intermediary product of fat metabolism. In the absence of carbohydrate this acid is only partially oxidised, aceto-acetic acid and acetone being formed.



Blum [1910] however maintains that normally butyric acid is first converted into aceto-acetic acid and that this is reduced to β-hydroxybutyric acid. Marriot [1914] also favours this view. In any case when carbohydrate is available the fatty acids are completely oxidised, carbon dioxide and water being the end products.

How does this carbohydrate act?

Ringer [1913] concludes from a series of very interesting experiments that it is not the whole carbohydrate molecule, but only the CHO radicle, that is necessary to prevent acidosis. He found that gluconic acid, differing only from glucose in having the CHO radicle replaced by CO.OH, did not prevent acidosis, while $CH_3 . CHO$ and $C_2H_5 . CHO$, when fed to phloridzinised dogs, brought about a great decrease in the total acetone output.

Ringer [Ringer, Frankel and Jonas, 1913] has also shown that isobutyric acid, when fed to phloridzinised dogs, gives rise to extra glucose and

diminished acidosis; whereas normal butyric acid does not give rise to glucose but to an increased acidosis. He suggests that a glucosidic union is accomplished between β-hydroxybutyric acid and glucose by means of the CHO radicle, and that the derivatives of normal butyric acid are in this way converted into iso-compounds. This would explain the seeming necessity of a constant fat : carbohydrate ratio.

But more than a mere chemical union between β-hydroxybutyric acid and glucose seems to be involved. This is suggested by the fact that the feeding of 30 g. of sugar is sufficient to reduce the total acetone of complete starvation from 200–300 mg. to 35 mg. (see Table XVI), while a further addition of 70 g. of sugar is necessary to reduce this to less than 10 mg. This reduction could not be due to a diminution of the quantity of acetone derived from protein, as the total nitrogen in the latter case was quite as high as in the former. Again, the addition of 60 g. of sugar to a fat diet reduced the total acetone from 818 mg. to 81 mg. (Tables XII and XIII) while with a further addition of 90 g. of sugar 24 mg. of total acetone were excreted.

Ringer, in his experiments already mentioned, administered acetaldehyde and propyl aldehyde to phloridzinised dogs on a constant diet. The result was that not only was the acetone in the urine greatly diminished, but there was an increase in the output of sugar. This increase was more than could be accounted for even if all the carbon in these aldehydes had been converted into glucose carbon. He concluded that this increase in glucose was due to some non-glucogenetic substance in the animal metabolism becoming glucogenetic owing to the influence of the CHO radicle. Perhaps the same change can take place when sugar is given in starvation. A small quantity of carbohydrate may be sufficient to bring about the necessary change, while a large amount may be unable to increase its extent, or, when the non-glucogenetic substance has become glucogenetic it may only remain in that condition so long as acidosis is present in a marked degree. It is very probable that this potential glucogenetic substance is either fat itself or one of the products formed during the early stages of fat catabolism.

This work was carried out under the direction of Dr E. P. Cathcart, to whom I offer my thanks.

The expenses in connection with this research were in part defrayed by a grant made to Dr E. P. Cathcart by the Carnegie Trust.

Conclusions.

1. The quantity of acetone bodies excreted by the normal individual, on an ordinary diet containing a sufficiency of carbohydrate, is influenced chiefly by the protein intake. On an ordinary diet 10–30 mg. are excreted daily.

2. The administration of quite small amounts of carbohydrate to the starving organism brings about a great reduction in the acidosis.

3. The administration of protein to the fasting organism causes a similar decrease, but this is neither so marked nor so rapid as in the case of carbohydrate.

4. The administration of glycerol under similar conditions also causes a very definite reduction in the acidosis.

5. The administration of fat to the starving organism increases the acidosis.

6. Administration of alcohol is without effect on the degree of acidosis.

7. The amount of acetone bodies in the urine during the first few days of starvation depends on the initial carbohydrate storage. These substances appear in abnormal amounts immediately the ratio of the fat to the carbohydrate burnt becomes greater than about 2 : 1. Immediately the ratio becomes less than this the acetone output is reduced to normal.

8. While a relatively large amount of carbohydrate is required to prevent acidosis, quite a small amount suffices to check it very markedly.

9. When acetone bodies are excreted in excessive amounts they are for the most part derived from fat.

10. Some evidence is put forward in favour of the possibility of fat being converted into carbohydrate in the body.

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XLV. THE FIXATION OF SALVARSAN AND NEOSALVARSAN BY THE BLOOD AFTER INTRAVENOUS INJECTION.

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(*Received October 27th, 1915.*)

It was found by Nierenstein [1908] that when serum was shaken with atoxyl (sodium *p*-aminophenylarsenate) and the proteins subsequently precipitated from the mixture with tannic acid, the precipitate invariably contained arsenic which could not be removed by washing. He further observed that when derivatives of atoxyl were used instead of atoxyl itself, arsenic was present in the precipitate only in those cases in which the derivative contained a free amino or imino group, whereas negative results were obtained with compounds such as benzoyl-acetyl-atoxyl, in which both hydrogens of the amino group were substituted, and with sodium *p*-hydroxyphenylarsenate, in which the amino group of atoxyl is replaced by hydroxyl.

In each case in which arsenic was present in the tannic acid precipitate the "atoxyl serum" could not be freed from arsenic by continued dialysis.

Breinl and Nierenstein [1908, 1909, 1 and 2] found that the same occurred when atoxyl and its derivatives were injected into animals. Arsenic was found in the serum only after the injection of compounds containing free amino or imino groups. It was concluded from these experiments that atoxyl combines with the serum proteins by means of its amino group.

The experiments described below were performed to ascertain whether similar combinations could be obtained with salvarsan and neosalvarsan. As these compounds are much less stable in solution than atoxyl, it was not possible to carry out any experiments in vitro, and only experiments with living animals were made.

For the detection of arsenic, the serum was decomposed with a mixture of sulphuric and nitric acids, all trace of nitric acid removed by continued evaporation, and the residue tested for arsenic by the modification of Gutzeit's method recommended by Sanger and Black [1907]. Care was taken to employ reagents containing no arsenic.

The Fixation of Salvarsan and Neosalvarsan by Serum.

Goats were injected intravenously with salvarsan or neosalvarsan and blood was drawn after various intervals of time. The blood was allowed to clot and the serum poured off and filtered. The serum was then dialysed in parchment bags against running water or in some cases against running saline to prevent precipitation of the globulins.

Dialysis was continued until all dialysable arsenic was eliminated from the serum, which was ascertained by dialysing for 12 hours against a litre of distilled water or saline, evaporating the dialysate to a small bulk and testing for arsenic.

The dialysed serum was then filtered and 50 cc. decomposed and tested for arsenic. In every case arsenic was found in the serum. In some cases a portion of the dialysed serum was precipitated with 2 % tannic acid solution, the precipitate was well washed and decomposed and tested for arsenic with a positive result.

These experiments show that after injections of salvarsan and neosalvarsan, some arsenic is bound to the serum proteins in a form which cannot be removed by dialysis.

A control experiment was performed in which a very dilute solution of neosalvarsan was dialysed into running water for some days and the residue, on decomposing, was found to contain no arsenic.

It was found that continued dialysis was necessary before the dialysate was obtained free from traces of arsenic, and in some cases traces dialysed out even after more than seven days. It seemed probable that the combination with the serum slowly decomposed.

(1) *Goat* 1. Injected intravenously with 0·3 g. salvarsan. After 24 hours it was bled, the serum separated and 80 cc. dialysed as above.

Serum before injection—arsenic absent.

After injection 40 cc. dialysed serum—arsenic present; 40 cc. precipitated with tannic acid—arsenic present in ppt.

(2) *Goat* 2. 0·9 g. neosalvarsan was injected intravenously and serum obtained after 7 hours and 24 hours; both dialysed as before.

Serum before injection—arsenic absent.

7 hours after injection—tannic acid ppt. from 50 cc. serum—arsenic present.

24 hours after injection 50 cc. serum used—arsenic present.

(3) *Goat* 3. 0·75 g. neosalvarsan injected intravenously, bled after 12 hours and serum dialysed.

Serum before injection 50 cc.—arsenic absent.

Serum after injection 50 cc.—arsenic present.

(4) *Goat* 4. 0·8 g. neosalvarsan injected intravenously, bled after 16 hours and serum dialysed.

Before injection 50 cc. serum—arsenic absent.

After injection 50 cc. serum—arsenic present.

An experiment was also performed to see if inorganic arsenic was bound in the serum in a similar manner. A goat was injected intravenously with an amount of arsenious acid corresponding with 0·6 g. neosalvarsan, dissolved in sodium hydrate and diluted with saline. After 24 hours blood was drawn, and the serum dialysed. 100 cc. of serum after dialysis gave no arsenic test. The same goat was subsequently injected intravenously with 0·6 g. neosalvarsan and bled after 24 hours. The serum after dialysis gave a positive arsenic reaction.

Time required for the arsenic to be completely eliminated from the blood.

Many investigations have been made on the rate of elimination of arsenic from the body after injections of salvarsan. Amongst the more recent may be mentioned that of Beveridge and Walker [1911], who found that arsenic could be detected in the urine of rabbits up to the eleventh day after intravenous injection of salvarsan and neosalvarsan. Stümpke and Siegfried [1911] claimed that traces of arsenic were found in the urine of men and other animals even after several months had elapsed, but they were unable to demonstrate the presence of arsenic in the blood later than 24 hours after intravenous injection, and they suggested that secondary depôts of arsenic were formed in the organs and then gradually excreted in the urine.

Schütte [1912] stated that with horses no arsenic could be detected in the urine and faeces later than 13 days after intravenous injection of salvarsan.

Stühmer [1914] investigated the properties of serum drawn at increasing intervals after intravenous injection of salvarsan and neosalvarsan to ascertain how long after injection it was possible to detect specific therapeutic properties in the serum. He compared the different sera with regard to their protective

action against trypanosome infection of mice, and at the same time tested the sera for the presence of free salvarsan by the Ehrlich-Bertheim reaction. The solution employed for this reaction is made by dissolving *p*-dimethyl-aminobenzene in concentrated hydrochloric acid, adding excess of mercuric chloride and dissolving any precipitate which forms by the addition of a few drops of hydrochloric acid. With this solution salvarsan gives an orange precipitate.

Stühmer found that there was a parallelism between the protective action of the sera against trypanosomes and the intensity of the Ehrlich-Bertheim reaction. Serum drawn as late as the seventh day after injection of salvarsan postponed the infection. This action was increased when the serum was heated to 56° C. and the intensity of the Ehrlich-Bertheim reaction was also increased. With neosalvarsan after the second day the serum gave no reaction either chemically or biologically. Thus after two days no free neosalvarsan appeared to be present in the serum.

Riebes [1914], employing Abelin's [1912] diazo reaction with resorcinol for detecting the amino group of salvarsan, found that in the majority of cases the reaction disappeared from serum three hours after the injection.

From these experiments it appears that free salvarsan and neosalvarsan are eliminated in a very few days from the blood.

The following experiments were made to ascertain how long a time after injection "bound arsenic" could be detected in the serum. The serum drawn at different intervals was tested for free salvarsan by the Ehrlich-Bertheim reaction, it was then dialysed into running water and the residue decomposed and tested for arsenic as before. It was found almost impossible to see the orange precipitate or coloration in the presence of the serum when very little neosalvarsan was present, the serum was therefore dialysed into water and the reagent added to the latter.

0·9 g. neosalvarsan was injected intravenously into a goat. 50 cc. serum were used in each case.

TABLE I.

Time after injection	As in dialysed serum	E.-B. reaction
7 hours	+	+
24 „	+	Faint colour
2 days	+	„
6 „	+	–
19 „	trace	–
28 „	–	–

After 19 days therefore a trace of bound arsenic was still detected in the serum, whereas a faint reaction for salvarsan was only obtained after two days.

In another experiment, Table II, 0·8 g. neosalvarsan was injected intravenously.

TABLE II.

Time after injection	As in dialysed serum	E.-B. reaction
16 hours	+	+
24 „	+	faint colour
48 „	+	–
6 days	+	–
21 „	trace	–

In this experiment a trace of arsenic was still found in the dialysed serum after twenty-one days.

From these results it is seen that the arsenic which is bound in the blood is only very slowly eliminated, whereas the bulk of the neosalvarsan is quickly excreted, only a trace being present after the first twenty-four hours.

Distribution of "bound arsenic" in the blood.

The following experiment was carried out to ascertain in what parts of the blood arsenic was retained.

A goat which had received 0·8 g. neosalvarsan was bled after 7 days so that all free neosalvarsan should be excreted. 150 cc. of blood were defibrinated, the red cells separated by centrifugalisation and repeatedly washed in the centrifuge with normal saline. The final washings from the red cells were found to be free from arsenic. The fibrin after washing was free from arsenic. Arsenic was present in the red blood cells and also in the plasma after dialysis.

SUMMARY.

After intravenous injection of goats with salvarsan and neosalvarsan, the serum contains arsenic in a form which cannot be separated from the proteins by dialysis, and which is precipitated with the serum proteins by tannic acid. Salvarsan and neosalvarsan behave, therefore, in a similar manner to atoxyl.

No such combination is obtained when inorganic arsenic is injected.

This combined arsenic is found in the blood long after all free salvarsan and neosalvarsan have been eliminated.

This combined arsenic is found in the plasma and in the red blood cells, but no trace of arsenic is retained in the fibrin.

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XLVI. THE EFFECT OF INGESTION OF UREA, SODIUM LACTATE AND SODIUM BICARBONATE ON THE REACTION OF THE BLOOD AND THE COMPOSITION OF THE ALVEOLAR AIR IN MAN.

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(*Received October 29th, 1915.*)

The present paper records an attempt to test the question whether certain substances taken by the mouth have the effect of making the blood in the body more alkaline.

Some preliminary experiments on the subject were made by Wolf and Barcroft [1915], who in a few experiments on dogs and on Barcroft himself found that blood exposed to oxygen at 17 mm. pressure took up somewhat more of the gas after ammonium citrate or urea had been taken. This suggested increased alkalinity of the blood.

Toyojiro Kato [1915, 1] recently observed that addition of certain alkaline substances to the blood, NaOH, Na_2CO_3, $NaHCO_3$, Na_2HPO_4, accelerates the oxidation and retards the reduction of blood. Accepting this, one would expect that alkali would increase the percentage saturation of oxygen in blood exposed to a given oxygen pressure, just as acid reduces the percentage saturation [Barcroft and colleagues, 1914]. We have then used this measurement as a test for the changed reaction.

The method of exposing blood to a standard gas mixture is that described by Kato [1915, 2], in which 1–2 tenths of a cc. is put into the bulb of a small pipette, gas is then passed over it from a gas holder, the pipette being rotated in a water bath at 37°–38° C. for about ten minutes. This suffices to secure

complete equilibrium between the blood and the gas. The blood is then transferred to the differential gas analysis apparatus designed by Barcroft [1914, p. 300] for the analysis of 0·1 cc. of blood. In this its percentage saturation is determined.

On account of the novelty of Kato's tonometer I thought it desirable to test the method rigorously. The following are the results of two sets of determinations in which I tried to see whether on repeated analysis the same blood yielded similar values for the percentage saturation.

Percentage Saturation of Oxygen in Blood.

No. of exp.	Number of determinations	Animal	Maximal result	Minimal result	Mean
I	7	Sheep	58·8	53·3	55·3
II	7	Rabbit	43·8	39·8	41·1

To these results I added some on the percentage saturation of Barcroft's normal blood when exposed to 17 mm. O_2 pressure. They are as follows:

Experiment III.

	Percentage saturations				
(1)	77·1	75·1	79·6	—	mean 77·3
(2)	74·5	71·7	67·8	—	„ 71·3
(3)	71·0	74·0	73·4	73·9	„ 73·8
(4)	67·6	67·6	71·7	—	„ 69·1

These results shew that, so far as the method of gas analysis is concerned, any individual sample of blood gives very concordant results. They suggest that the blood of the same person varies to a trifling extent from day to day. This result must however be received with great caution, lest it should be due to a slight want of uniformity in the gas used on different occasions.

Results on my own blood were slightly more uniform:

Experiment IV.

	Percentage saturations		
(1)	72·8	72·5	mean 72·7
(2)	73·1	69·6	„ 71·4
(3)	74·0	72·1	„ 73·0

In no case do the above observations include cases in which substances for investigation such as urea, sodium lactate etc. have been taken by the patient so recently as the day before the observation.

Experiments on the percentage saturation of human blood at 17 mm. O_2 pressure.

The following experiments shew that urea taken by the mouth causes the blood to become more alkaline, i.e. causes the percentage saturation in the blood to rise when exposed to the standard mixture.

Effect of Urea on Percentage Saturation of Blood.

		Percentage saturation				
			After			
					Approximate time	
No. of exp.	Dose	Before	1 hour	2 hours	5 hours	Next day
V	15 g. urea in 250 cc. water	77·3	82·0	—	84·2	—
VI	„	71·3	—	—	76·7	72·0
VII	„	73·1	—	80·2	—	—

Poulton and Ryffel [1913], who performed experiments on the percentage saturation of blood in uraemic patients, state that the addition of urea, in small concentrations, to blood does not change its affinity for oxygen. This I verified:

Experiment VIII. Rabbit's Blood.

	Urea added to 1 cc. blood	Percentage saturation with oxygen at 17 mm.
(1)	Nil	48·8 Mean of 2 observations
(2)	0·1 cc. N/10 CON_2H_4	48·1 „ „ „
(3)	0·2 cc. „	47·1 „ „ „
(4)	0·3 cc. „	49·4 „ „ „

The solution of urea was normal in reaction. Since the urea itself does not affect the affinity of blood for oxygen, the inference is that urea taken by the mouth becomes partially converted into ammonia in the body [Wolf, 1912].

Exp. IX. It therefore became of interest to ascertain the concentration of ammonia, which, if added directly to blood, would produce the same change as the urea which was given by the mouth. This proved to be 0·2 cc. of N/10 NH_3 per cc. of blood or 0·017 %. This quantity changed the percentage saturation at 17 mm. from 73 to 83·7 %. (Mean of two determinations: 81·8 and 85·5.)

Sodium lactate was suggested by Sir Almroth Wright on the ground of its rapidly causing the urine to become alkaline. It does not follow that

the blood would become appreciably alkaline for the sodium might be rapidly eliminated.

The preparation of sodium lactate which we used was slightly acid in reaction and when added to defibrinated blood it caused the percentage saturation at 17 mm. O_2 to drop, when added in dilute solutions; after 0·2 cc. N/10 lactate further addition had no effect, presumably because the degree of ionisation does not increase.

Experiment X. Rabbit's Blood.

	Sodium lactate added to 1 cc.	Percentage saturation with oxygen at 17 mm.	
(1)	Nil	48·8	Mean of 2 determinations in each case
(2)	0·1 cc. N/10 $NaC_3H_5O_3$	41·8	„ „ „
(3)	0·2 cc. „ „	32·2	„ „ „
(4)	0·3 cc. „ „	32·9	„ „ „

Given by the mouth however sodium lactate has precisely the opposite effect.

Experiments XI, XII and XIII. Effect of Sodium lactate.

		Percentage saturation with oxygen					
			After				
No. of exp.	Dose	Before	1 hour	2 hours	3 hours	4 hours	Next day
XI	5 g. in about 10 cc. of water	72·7*	74·7	83·9	78·6	—	—
XII	„	74·4	75·5	—	77·5	72·5	72·6
XIII	„	72·6	76·2	88·0	74·1	75·0	—

* Each of these results is the mean of two determinations.

These figures indicate that the maximal effect is obtained at the end of about two hours. The question then arises, to how much alkali does this correspond? On this point I obtained the following data by the addition of NaOH directly to defibrinated blood.

Experiment XIV. Addition of NaOH to Human Blood (Barcroft).

	NaOH added to 1 cc. of blood	Percentage saturation with oxygen at 17 mm.	
(1)	Nil	69·3	Mean of 8 determinations
(2)	0·1 cc. N/10 NaOH	80·1	„ 4 „
(3)	0·2 cc. „	80·7	„ 3 „

The effect of adding urea, sodium hydroxide, sodium lactate and ammonia to defibrinated blood is shewn in Fig. 1.

A third substance which I tried was sodium bicarbonate.

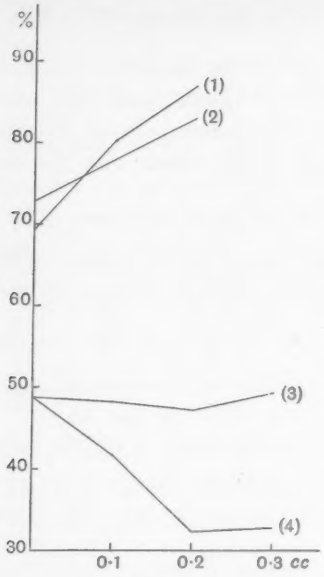

Fig. 1.

Ordinates = Percentage saturation of blood at 17 mm. oxygen pressure.
Abscissae = Quantity of drug added.

(1) N/10 NaOH (Blood of Barcroft)
(2) N/10 NH_3 („ Momose)
(3) N/10 CON_2H_4 (Blood of Rabbit)
(4) N/10 $NaC_2H_5O_2$ („ „)

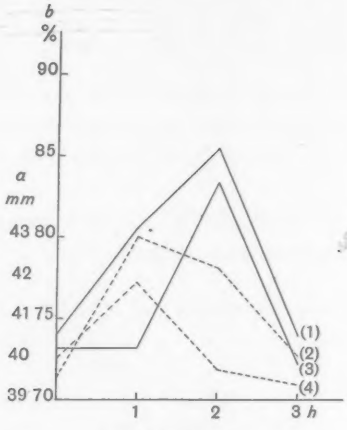

Fig. 2.

(3), (2). Exp. XVII.
(1), (4). Exp. XVIII.

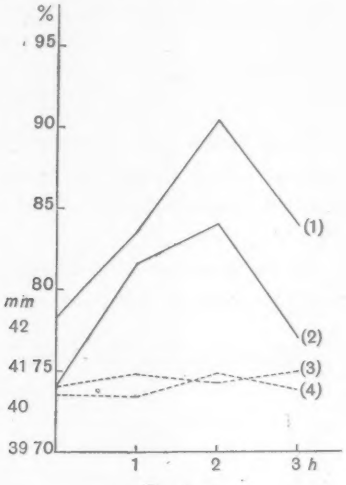

Fig. 3.

(1), (4). Exp. XIX.
(2). (3). Exp. XX.

Ordinates: (*a*) mm. pressure of CO_2 (dotted line).
(*b*) Percentage saturation of blood at 17 mm. oxygen pressure (continuous line).
Abscissae = Time after taking dose in hours.

It might be expected that the addition of substances which would raise the percentage saturation at 17 mm. would also raise the alveolar carbonic acid. I made numerous measurements of the alveolar CO_2, therefore, before and after taking sodium lactate with the following results:

Alveolar CO_2 pressure in mm. (Momose).

No. of exp.	Dose of sodium lactate	Before	After 1 hour	After 2 hours	After 3 hours
XV	5 g.	40·1	40·9	40·1	40·2
XVI	„	40·2	42·4	41·0	40·5

The change in the alveolar CO_2 is extremely slight, but such as it is it reaches a maximum at the end of the first hour.

In order to see whether the change in the CO_2 really reaches its maximal value in a different time from the change in the percentage saturation at 17 mm. a series of observations of the two were undertaken concurrently in the same experiment.

Effect of Sodium lactate on Percentage Saturation and Alveolar Carbonic Acid.

No. of exp.	Dose of sodium lactate		Before	After 1 hour	After 2 hours	After 3 hours
XVII	5 g.	% sat.	73·1	73·1	83·3	72·2
		Alv. CO_2 mm.	39·5	43·0	42·3	40·1
XVIII	„	% sat.	74·0	80·5	85·3	74·0
		A.v. CO_2 mm.	40·1	41·9	39·7	39·4

These figures bear out what I found in the earlier observations, namely, that the trifling change in CO_2 pressure is most evident at the end of the first hour and the change in percentage saturation at the end of the second (Fig. 2).

It was a matter of some interest to ascertain whether a similar result would be obtained when sodium bicarbonate was taken (Fig. 3).

Effect of Sodium bicarbonate on Percentage Saturation and Alveolar Carbonic Acid.

No. of exp.	Dose of $NaHCO_3$		Before	After 1 hour	After 2 hours	After 3 hours
XIX	4 g. (3·8 g. equivalent to 5 g. sodium lactate)	% sat.	78·2	83·5	90·3	83·9
		Alv. CO_2 mm.	40·4	40·3	40·9	40·5
XX	„	% sat.	73·9	81·5	83·9	76·9
		Alv. CO_2 mm.	40·6	40·9	40·7	41·0

Conclusions.

Two points seem to be established.

(1) Urea, sodium lactate and sodium bicarbonate, in the doses taken, produce little change on the alveolar CO_2.

(2) These substances produce an appreciable change on the affinity of blood for oxygen in the presence of CO_2.

It remains to investigate the question: Do these compounds affect the percentage saturation of the blood with oxygen in the presence of the alveolar pressure of CO_2? So far as sodium lactate in 5 gram doses is concerned the effect does not appear to be appreciable:

Effect of Sodium lactate on Percentage Saturation in the Presence of 27·7 mm. of Oxygen and 40 mm. CO_2.

No. of exp.	Dose	Before	After 2 hours
XXI	5 g. sodium lactate	57·4 (Alv. CO_2=40)	58·5 (Alv. CO_2=39)
XXII	„	47·1 (Alv. CO_2=40)	45·8 (Alv. CO_2=39)

I am indebted with warmest thanks to Mr Barcroft for his kind guidance in the performance of this investigation.

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XLVII. THE VOLUMETRIC ESTIMATION OF TOTAL SULPHUR AND SULPHATES IN SMALL QUANTITIES OF URINE.

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(*Received November 8th, 1915.*)

Introductory.

A large number of publications have appeared from time to time upon the estimation of sulphates by the benzidine method of Raschig [1903]. The majority, if not all, of these communications have declared that, in general inorganic analysis, the method is one on which the utmost reliance is placed.

The method, which has as its basis the fact that benzidine sulphate is very insoluble in water, depends upon the precipitation of the sulphate present by means of a dilute solution of benzidine hydrochloride. The insoluble benzidine sulphate is then removed by filtration, and can be accurately titrated against standard alkali, as benzidine salts are readily dissociable. The application of the method to the analysis of biological material was not attempted until two papers appeared, almost simultaneously, by Rosenheim and Drummond [1914][1], and Gauvin and Skarzynski [1913].

Both these papers dealt with the analysis of urine by this method. That of Rosenheim and Drummond contained a systematic investigation into the conditions necessary for accurate analyses to be made, and showed that the inorganic and ethereal sulphates in urine could be rapidly and accurately determined by the benzidine method. These authors also suggested that the method could be adapted for the analysis of very small quantities of urine and for the determination of total sulphur.

[1] Rosenheim and Drummond's results were communicated to the Biochemical Society at a meeting held on November 20th, 1913.

This was partially borne out by the independent findings of Gauvin and Skarzynski, who showed that, by applying the benzidine method to the solution of salts obtained after oxidising the sulphur in urine by various fusion methods, analyses of the total sulphur present might be made.

These two authors also confirmed the results of Rosenheim and Drummond for the analysis of the total and inorganic sulphates in urine, although they did not, apparently, investigate so closely the conditions necessary for accuracy.

Subsequently Raiziss and Dubin [1914] made use of the benzidine method in the determination of total sulphur in small quantities of urine, and in their communication made some criticisms of the titration of the benzidine sulphate with alkali.

These workers stated that the method was not trustworthy for the analysis of small quantities of urine, as they found that the quantity of sulphuric acid liberated from 10 cc. of urine was too small to be accurately titrated with 0·1 N alkali, and further, they were unable to obtain a sharp end-point to the titration, except after repeated additions of alkali and boilings. To surmount these apparent disadvantages, they devised an alternative method of titrating the benzidine sulphate, which was based on an observation that benzidine sulphate may be quantitatively oxidised by means of potassium permanganate.

Their paper only deals with the analysis of the total sulphur in urine by this method, applying the precipitation of the benzidine sulphate to the final solution of salts obtained after oxidising the urine by the method of Benedict [1909], as modified by Denis [1910]; and they claim considerable accuracy for the method even in the analysis of as small a volume as 2 cc. of urine. The present communication records the results of a series of experiments which were carried out to test whether the earlier method might not be used successfully for the micro-estimation of the sulphates in urine and the total sulphur in biological material in general.

Incidentally, the method devised by Raiziss and Dubin was investigated, mainly with the object of determining whether any great advantage lay in the titration of the benzidine sulphate with permanganate, which they advocate, instead of with alkali. Naturally, the much larger titration figure, which is obtained in the permanganate method, is a distinct advantage when small quantities of benzidine sulphate are being dealt with, but I have not been convinced that this is in itself sufficiently important to necessitate an alteration in the method.

The objections raised by the American workers to the titration of the benzidine sulphate with alkali appear to have little foundation, for, first, I have never yet, in the course of many hundred urinary analyses by this method, encountered an indefinite end-point to a titration; provided that during the filtration strict care is paid to certain details of technique. If the precipitated benzidine sulphate is allowed to suck dry upon the filter, it will form a glistening layer which will not disintegrate upon suspension in water, but will float about in the liquid in the form of small flakes, which are not readily attacked by the alkali during titration. Should the precipitate be once obtained in this form, an end-point by direct titration with alkali is only possible after "repeated boilings and additions of alkali." However, even in this case, a rapid and accurate titration may be made by dissolving the precipitate in an excess of standard alkali by warming, and then titrating that excess with standard acid.

The great importance of the details of technique, which must be observed during filtration, have been emphasised by almost all the investigators who have used the method; hence it is possible that Raiziss and Dubin failed to carry out the filtration in an entirely satisfactory manner.

Their other objection to the titration of the benzidine sulphate with alkali, namely, that 10 cc. of urine yields too small a quantity of sulphuric acid to titrate accurately with standard alkali, receives its answer in the results recorded in the present paper, which show that by the employment of a more dilute alkali, 0·02 N, very accurate analyses of much smaller volumes of urine than 10 cc. may be made.

The method employed by Raiziss and Dubin is as follows. The precipitated benzidine sulphate is filtered off upon an asbestos filter, and is then dissolved in a comparatively large volume of dilute sodium hydroxide, by warming. The solution thus obtained is cooled to room temperature, and, by the addition of a certain volume of concentrated sulphuric acid, is brought to an empirical temperature at which titrations are made with standard potassium permanganate.

In order to standardise the permanganate solution, and obtain its value in terms of sulphur, they carried out estimations upon a pure sulphuric acid solution, and they thus arrived at the factor 0·000099 to represent the value of 1 cc. 0·1 N potassium permanganate in grams of sulphur. Utilising this factor they calculate their results for the analysis of urine.

Upon trying the method, as described by them, I found that in the analysis of pure solutions of sulphates there is no difficulty in the technique. It was

my experience, however, that the end-point here really does leave much to be desired. This is particularly the case when one is titrating comparatively large quantities of benzidine sulphate.

The titration fluid passes through various shades of orange and yellow, until shortly before the end-point is reached one has an almost colourless solution, and I have often found it most difficult to determine precisely when I have obtained the permanent pink coloration, lasting for twenty seconds, which is advised by the authors.

The empirical temperature of the titration seems an unsatisfactory condition, and, in my experience, unreliable results may occur if one does not always titrate at the same temperature.

TABLE I.

Solution analysed	Cc. analysed	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·1 N $KMnO_4$	% S	Factor (g. of S equivalent to 1 cc. $KMnO_4$)
Solution 1	20	0·2402	—	—	0·164	—
0·1 N H_2SO_4	„	0·2394	—	—		—
	10	—	9·99	—		—
	„	—	9·99	—		—
	„	—	9·99	—	0·1602	—
	„	—	10·00	—		—
	„	—	10·02	—		—
	0·5	—	—	8·44	—	
	„	—	—	8·55	—	
	„	—	—	8·49	—	0·0000944 g. S
	„	—	—	8·57	—	
	„	—	—	8·52	—	
Solution 2	50	0·0918	—	—	0·0249	—
Sodium	„	0·0907	—	—		—
sulphate	25	—	3·91	—		—
containing	„	—	3·92	—		—
0·0245 % S	„	—	3·91	—	0·0250	—
	„	—	3·93	—		
	„	—	3·90	—		—
	2	—	—	5·39	—	
	„	—	—	5·30	—	
	„	—	—	5·30	—	0·0000939 g. S
	„	—	—	5·42	—	
	„	—	—	5·30	—	
Solution 3	20	0·0216	—	—	0·0148	—
Sodium	„	0·0216	—	—		—
sulphate	100	—	8·88	—		—
containing	„	—	8·91	—	0·0143	—
0·0145 % S	„	—	8·92	—		—
	„	—	8·90	—		—
	5	—	—	7·73	—	
	„	—	—	7·70	—	0·0000937 g. S.
	„	—	—	7·75	—	
	„	—	—	7·72	—	

As a result of the analysis of many pure solutions of sulphates, I have not obtained a constant value to express the strength of the permanganate solution in terms of sulphur. The factor is an empirical one and considerably influenced by the experimental conditions. As a rule I have obtained somewhat lower figures than the 0·000099 given by Raiziss and Dubin. In the analysis of urine by this procedure, good results can be obtained if the permanganate is standardised against a pure sulphate solution under certain experimental conditions, and the urinary analyses are then conducted under identical conditions. The method, however, is in no way superior to the usual process, in which the benzidine sulphate is titrated directly in aqueous suspension with standard alkali.

Some of the analyses obtained upon pure sulphate solutions may be recorded here (Table I).

Having come to the conclusion that the permanganate titration suggested by Raiziss and Dubin possessed no advantages over the alkali titrations used by earlier authors, it was then necessary to determine first, whether the alkali titration process was adaptable to the estimation of small quantities of sulphates, and if so, to apply it to the estimation of the sulphates in small volumes of urine; and secondly, how best to utilise the process in the estimation of total sulphur.

Experimental.

1. The Estimation of small quantities of Sulphates in pure solution by the Benzidine method.

In the first place, it was found that the precipitation of very small quantities of sulphates from pure solutions as benzidine sulphate was not quantitative unless the reaction of the fluid was carefully adjusted. Rosenheim and Drummond pointed out that an excess of hydrochloric acid prevented the quantitative precipitation of benzidine sulphate, and it was found that only a faint acid reaction must be present during precipitation. If this is so the benzidine sulphate separates quantitatively. The separation of the small precipitates thus obtained was attempted by several means, such as centrifugalisation, but it was finally found that the simple filtration method could not be improved upon. Naturally, the greatest care must be paid to the details of technique during filtration when very small precipitates are dealt with. The details are as follows:

A 5 cm. funnel is fitted into the neck of a 250 cc. filtering flask and carries

a small perforated porcelain filtering disc of 1 cm. diameter. A suspension of filter paper pulp is poured into the funnel and sucked dry upon the porcelain disc by means of a filter pump. The layer of paper should not be too thick; 1–2 mm. is sufficient to retain benzidine sulphate crystals. The benzidine sulphate precipitate can now be directly poured on to the filter, and filtered by the application of a partial vacuum. Care must be taken to prevent the precipitate at any time sucking dry upon the filter, otherwise, as previously stated, it will be obtained in a flaky condition and will not be attacked by the alkali during the titration [Raschig, 1903; Järvinen, 1913]. The pressure is therefore gently released at the moment before the precipitate sucks dry upon the filter. The vessel in which the precipitation has been carried out is then washed out into the funnel with two washings of about 2–3 cc. of a saturated solution of benzidine sulphate in distilled water, and the washings drawn through the filter by gentle suction, again taking care to prevent the precipitate being sucked dry. It is not advisable, especially when dealing with small precipitates, to wash the precipitate with distilled water, as do Raiziss and Dubin, because of the slight, but appreciable solubility of benzidine sulphate in water.

The precipitate, together with the filter paper and porcelain disc, are transferred by means of a clean glass rod into the vessel in which the precipitation was carried out, and the rod and funnel washed into it with a fine jet of distilled water.

The suspension of benzidine sulphate and filter paper, which, provided care has been taken during filtration, should show no flakes of benzidine sulphate, is then heated to boiling-point and titrated whilst hot with 0·02 N potassium hydroxide. As an indicator a dilute solution of phenolphthalein, or a drop or two of a saturated alcoholic solution of methyl red, can be used. Both indicators yield sharp end-points.

It is always necessary, when titrating benzidine sulphate precipitates with 0·02 N alkali, to carry out a blank estimation upon the reagents for each series of analyses.

Should the benzidine sulphate precipitate be obtained upon the filter in the form of flakes which cannot be titrated with alkali, all that is necessary to obtain an accurate result is to dissolve the precipitate in an excess of standard alkali by warming, and then to titrate the excess of alkali with standard acid, using either of the indicators mentioned above.

The following results, which have been obtained upon a pure solution of sodium sulphate, demonstrate the accuracy of the method.

TABLE II.

Analysis of a Solution of Sodium sulphate containing 0·0248 % *S.*

Cc. analysed	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% S
50	0·0914	—	—	0·0250
,,	0·0910	—	—	
25	—	3·91	—	
,,	—	3·92	—	0·0251
,,	—	3·91	—	
,,	—	3·93	—	
10	—	—	8·04	
,,	—	—	7·93	
,,	—	—	8·00	0·0253
,,	—	—	7·96	
,,	—	—	7·96	
5	—	—	3·94	
,,	—	—	3·88	
,,	—	—	3·92	0·0249
,,	—	—	3·94	
,,	—	—	3·86	
2	—	—	1·56	
,,	—	—	1·58	
,,	—	—	1·59	0·0252
,,	—	—	1·56	
,,	—	—	1·60	

Having determined definitely that upon pure solutions the method yielded highly accurate results, the application was extended to the micro-analysis of the urinary sulphates.

2. Estimation of small quantities of Urinary Sulphates.

2 *a*. *Inorganic sulphates.* The estimation of the inorganic sulphates in urine presented no difficulty whatever, it being quite sufficient to apply on a smaller scale the method as described by Rosenheim and Drummond for 20 cc. of urine. Accordingly the following technique was adopted and has yielded very accurate figures:

5 cc. of urine are measured into a 50 cc. beaker, acidified with 0·5 cc. of a 1 in 4 solution of hydrochloric acid (1 part conc. HCl to 3 of H_2O), and 20 cc. of the benzidine reagent are then added. After allowing the precipitate to settle for five minutes, it is filtered off upon a small filter-paper pulp filter in exactly the same way as has just been described for the estimation of pure sulphate solutions. The precipitate is transferred to the original beaker, and titrated at boiling-point with 0·02 N potassium hydroxide, using either phenolphthalein or methyl red as an indicator.

Quantities of urine as small as 2 cc. may be estimated by an exactly similar procedure, using proportional quantities of reagents. Analyses of the inorganic sulphates in two samples of urine are here shown. The control estimations were in all cases made by Folin's gravimetric method [1906].

TABLE III.

Sample	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
Urine 1	20	0·1069	—	—	0·183
	,,	0·1067	—	—	
	20	—	9·10	—	
	,,	—	9·00	—	
	,,	—	9·10	—	0·182
	,,	—	9·15	—	
	,,	—	9·12	—	
	10	—	4·55	—	
	,,	—	4·55	—	0·182
	,,	—	4·55	—	
	,,	—	4·55	—	
	5	—	—	11·55	
	,,	—	—	11·37	
	,,	—	—	11·25	
	,,	—	—	11·45	0·183
	,,	—	—	11·40	
	,,	—	—	11·46	
	,,	—	—	11·42	
Urine 2	20	0·0916	—	—	0·157
	20	—	7·70	—	
	,,	—	7·62	—	0·153
	,,	—	7·66	—	
	,,	—	7·60	—	
	10	—	3·88	—	
	,,	—	3·88	—	0·154
	,,	—	3·80	—	
	,,	—	3·82	—	
	5	—	—	9·76	
	,,	—	—	9·46	
	,,	—	—	9·79	0·154
	,,	—	—	9·56	
	,,	—	—	9·59	
	2	—	—	3·85	
	,,	—	—	3·87	
	,,	—	—	3·96	0·157
	,,	—	—	4·00	
	,,	—	—	4·00	

2 *b*. *The estimation of total sulphates.* This presented more difficulties than did the determination of the inorganic sulphates, mainly because the most careful regulation of the acidity after hydrolysis had to be made, in order that the precipitation of the benzidine sulphate should be complete.

In the earlier analyses, this was carried out as described by Gauvin and Skarzynski [1913], who, after hydrolysis of the ethereal sulphates in the usual manner, neutralised their solution with alkali and then reacidified it with the correct amount of hydrochloric acid. This procedure yielded fairly good results, but it was found, during some preliminary experiments, that there was no need to go to this trouble, as the ethereal sulphates in urine may be quantitatively hydrolysed by a far smaller proportion of hydrochloric acid than is usually employed.

Equal volumes of a sample of urine were hydrolysed by boiling with varying proportions of 1 in 4 hydrochloric acid, under identical conditions for 30 minutes. The analyses of total sulphates made on each sample, which are given below, demonstrate fully that the ethereal sulphates present in 20 cc. of urine are as completely hydrolysed by boiling for half an hour with 2 cc. of 1 in 4 hydrochloric acid as by boiling under identical experimental conditions with 15 cc. of the acid.

TABLE IV.

Urine hydrolysed for 30 minutes over constant source of heat.

Estimation	Cc. urine	Cc. 1 in 4 HCl	Weight of $BaSO_4$ in g.	% SO_3
Total sulphates	20	15	0·0824	0·142
	20	15	0·0828	
,,	20	10	0·0827	0·142
,,	20	10	0·0829	
,,	20	7	0·0826	0·142
,,	20	7	0·0835	
,,	20	5	0·0830	0·142
,,	20	5	0·0826	
,,	20	3	0·0837	0·143
,,	20	3	0·0835	
,,	20	2	0·0834	0·143
,,	20	2	0·0836	
Inorganic sulphates	20	—	0·0771	0·133
	20	—	0·0778	

This fact at once simplified the estimation of the total sulphates in urine on a small scale, and the method finally adopted as yielding the most reliable results is as follows.

5 cc. of urine are measured into a 25 cc. wide mouthed Erlenmeyer flask, and 0·5 cc. of a 1 in 4 solution of hydrochloric acid is added. A small glass

funnel inserted in the neck of the Erlenmeyer flask acts as a reflux trap to prevent spurting and undue concentration of the contents. Hydrolysis is then effected by boiling the liquid gently on a sand-bath for 30 to 40 minutes. The flask should be kept under observation during this period, and any appreciable diminution in the volume of the contents of the flask readjusted by the addition of a few drops of distilled water.

At the end of the period of time stated, the flask is removed from the sand-bath and allowed to cool. 20 cc. of the benzidine reagent are added, after the funnel and the neck of the hydrolysis flask have been washed down with a fine jet of distilled water. The precipitate is allowed to settle for five minutes, and is then filtered off and titrated in the manner already described.

The following urinary analysis will serve to show that the analysis of the total sulphates in as small a volume of urine as 2 cc. is rapidly and accurately made by this method.

TABLE V.

Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
20	0·0824	—	—	0·142
„	0·0824	—	—	
10	—	3·56	—	
„	—	3·57	—	0·142
„	—	3·54	—	
„	—	3·58	—	
5	—	—	8·78	
„	—	—	8·80	0·141
„	—	—	8·78	
„	—	—	8·79	
2	—	—	3·50	
„	—	—	3·49	0·140
„	—	—	3·46	
„	—	—	3·47	

2 *c*. *Pathological urines.* Many pathological urines have been analysed by the method, and, in all but a few cases, have yielded highly accurate results. The presence of pigments in some pathological urines may render the hydrolysed urine dark in colour, and the precipitated benzidine sulphate will then often carry down much of the coloration with it. Usually the benzidine sulphate precipitate thus obtained is of a pale mauve colour, and this coloration is apt to interfere somewhat with the end-point of the titration. No means has yet been found of preventing this, but personally I have found little difficulty in deciding the end-point in such cases, if phenolphthalein is used as the indicator, and one has a little experience of the method.

The presence of albumin at first presented difficulties in the analysis of some urines, but I have found that if the method recently described by Tracey and Welker [1915] for the removal of albumin from urine is used, accurate results may be obtained in these cases.

To determine this, a sample of urine was taken and divided into two portions. To one was added about 0·7 % of albumin in the form of a serum albumin solution, whilst to the other was added an equal volume of distilled water. The urine containing the albumin was then mixed with an equal volume of aluminium cream suspension, prepared as described by Tracey and Welker, and filtered, whilst the other specimen was diluted with an equal volume of distilled water. Upon analysis the two specimens yielded the following figures, which clearly demonstrate that the removal of albumin from urine by means of its absorption with aluminium cream does not affect the sulphate content of the fluid.

TABLE VI.

Urine	Estimation	Cc. taken	Cc. 0·02 N KHO	% SO_3
Treated	Inorganic sulphates	20	8·60	
		,,	8·25	0·067
		,,	8·34	
		10	4·17	
		,,	4·21	0·067
		,,	4·14	
Untreated	Inorganic sulphates	20	8·40	
		,,	8·25	0·067
		,,	8·20	
		10	4·20	
		,,	4·10	0·066
		,,	4·14	
Treated	Total sulphates	20	9·14	
		,,	9·12	0·073
		,,	9·10	
		10	4·55	
		,,	4·51	0·072
		,,	4·51	
Untreated	Total sulphates	20	9·22	
		,,	9·20	0·073
		,,	9·16	
		10	4·61	
		,,	4·51	0·073
		,,	4·50	

Some typical urinary analyses are here given, to show the high degree of accuracy attainable in analyses by this method.

TABLE VII.

Sample of urine	Estimation	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
Normal	Inorganic sulphates	20	0·0970	—	—	0·166
		10	—	4·16	—	
		,,	—	4·16	—	0·167
		,,	—	4·20	—	
		,,	—	4·17	—	
		5	—	—	10·20	
		,,	—	—	10·25	0·165
		,,	—	—	10·27	
		,,	—	—	10·27	
		2	—	—	4·18	
		,,	—	—	4·17	0·167
		,,	—	—	4·20	
		,,	—	—	4·18	
	Total sulphates	20	0·1062	—	—	0·182
		10	—	4·56	—	
		,,	—	4·56	—	0·182
		,,	—	4·55	—	
		,,	—	4·57	—	
		5	—	—	11·38	
		,,	—	—	11·35	0·181
		,,	—	—	11·33	
		,,	—	—	11·38	
		2	—	—	4·64	
		,,	—	—	4·68	0·187
		,,	—	—	4·70	
		,,	—	—	4·71	
Normal	Inorganic sulphates	10	—	4·61	—	
		,,	—	4·63	—	0·185
		,,	—	4·62	—	
		,,	—	4·66	—	
		5	—	—	11·78	
		,,	—	—	11·58	0·187
		,,	—	—	11·60	
		,,	—	—	11·62	
		2	—	—	4·78	
		,,	—	—	4·78	0·188
		,,	—	—	4·68	
		,,	—	—	4·68	
	Total sulphates	10	—	4·95	—	
		,,	—	4·89	—	0·196
		,,	—	4·88	—	
		,,	—	4·84	—	
		5	—	—	12·46	
		,,	—	—	12·38	0·198
		,,	—	—	12·50	
		,,	—	—	12·38	
		2	—	—	5·11	
		,,	—	—	5·08	0·204
		,,	—	—	5·12	
		,,	—	—	5·08	

TABLE VII (*continued*).

Sample of urine	Estimation	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% SO_3
Tubercular	Inorganic sulphates	10	—	—	8·74	
		,,	—	—	8·72	0·070
		,,	—	—	8·70	
		5	—	—	4·36	
		,,	—	—	4·31	0·069
		,,	—	—	4·40	
		,,	—	—	4·34	
	Total sulphates	10	—	—	9·95	
		,,	—	—	9·97	0·080
		,,	—	—	9·89	
		5	—	—	4·99	
		,,	—	—	4·96	0·080
		,,	—	—	4·95	
		,,	—	—	5·00	
Nephritis; albumin removed by aluminium cream	Inorganic sulphates	10	—	4·62	—	
		,,	—	4·61	—	0·195
		,,	—	4·64	—	
		5	—	—	11·51	
		,,	—	—	11·48	0·184
		,,	—	—	11·56	
		,,	—	—	11·49	
		2	—	—	4·60	
		,,	—	—	4·61	0·184
		,,	—	—	4·56	
	Total sulphates	10	—	5·25	—	
		,,	—	5·20	—	0·208
		,,	—	5·25	—	
		,,	—	5·18	—	
		5	—	—	13·00	
		,,	—	—	13·07	0·208
		,,	—	—	13·07	
		,,	—	—	13·01	
		2	—	—	5·20	
		,,	—	—	5·27	0·208
		,,	—	—	5·21	

3. The Determination of Total Sulphur.

Previous workers who have attempted to apply the benzidine method to the estimation of total sulphur have employed various oxidation methods; thus Gauvin and Skarzynski utilised various fusion methods, whereas Raiziss and Dubin employed the much more convenient process of Benedict, as modified by Denis [1910]. The American authors neutralised the final solution of salts obtained by this method, with sodium hydroxide, then

slightly re-acidified with hydrochloric acid and precipitated the sulphate present as benzidine sulphate in the usual manner.

The technique I have adopted for this determination resembles this method in its main points, and is as follows.

2 cc. of urine are measured into a 6 cm. porcelain evaporating basin, 0·5 cc. of Benedict's reagent is added, and the fluid evaporated gently to dryness upon the water-bath. The ignition of the residue is, however, carried out entirely by the use of a Barthel alcohol burner. This procedure was adopted as a result of some unpublished observations by Mrs M. C. Rosenheim.

TABLE VIII.

Cystine solution taken, cc.	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% S
5	0·0380	—	—	0·055
"	0·0381	—	—	
10	—	3·25	—	
"	—	3·25	—	
"	—	3·28	—	0·052
"	—	3·20	—	
"	—	3·20	—	
"	—	3·23	—	
2	—	—	3·28	
"	—	—	3·26	
"	—	—	3·23	0·0518
"	—	—	3·20	
"	—	—	3·24	
"	—	—	3·23	

She informed me that the variable amount of sulphur found by her in the blank estimations which she made on the purest reagents obtainable were traceable to the sulphur-containing impurities present in London coal gas. I have been able to corroborate this, and have found that if one uses reliable reagents, and conducts the ignition solely with the use of a spirit burner, the blank estimations seldom, if ever, contain detectable quantities of sulphur.

When the residue has been completely ignited over the spirit burner, it is allowed to cool, and 1 cc. of 1 in 4 hydrochloric acid is added. The dish is covered with a clock-glass, and is warmed upon the water-bath until the residue is entirely in solution. Should evaporation to dryness occur before this is the case, another 1 cc. of acid should be carefully added and the warming continued. When the whole of the black residue is in solution, the dish is allowed to remain upon the water-bath, the cover is washed into the dish and the contents gently evaporated to dryness. 1 cc. of distilled water is now added and the contents quantitatively transferred

to a 25 cc. beaker. 10 cc. of the benzidine reagent are then added to precipitate the sulphate present. The subsequent filtration and titration of the precipitate are carried out in the manner already described.

Analyses carried out upon a solution of pure cystine, for which I am indebted to Dr O. Rosenheim, are given in Table VIII, and are sufficient to show that the method is reliable.

0·1960 g. cystine was dissolved in 5 cc. of hydrochloric acid and diluted to 100 cc. with distilled water; the solution contained 0·052 % sulphur.

The results of the application of the method to the analysis of total sulphur in urine are also given.

TABLE IX.

Sample	Cc. urine	Weight of $BaSO_4$ in g.	Cc. 0·1 N KHO	Cc. 0·02 N KHO	% S
Urine 1	10	0·0481	—	—	0·066
	,,	0·0486	—	—	
	10	—	4·15	—	0·066
	,,	—	4·10	—	
	,,	—	4·17	—	
	,,	—	4·15	—	
	5	—	—	10·14	0·065
	,,	—	—	10·16	
	,,	—	—	10·10	
	,,	—	—	10·18	
	2	—	—	4·10	0·065
	,,	—	—	4·07	
	,,	—	—	4·11	
	,,	—	—	4·12	
Urine 2	10	0·0439	—	—	0·0604
	,,	0·0442	—	—	
	10	—	3·80	—	0·061
	,,	—	3·82	—	
	,,	—	3·80	—	
	,,	—	3·75	—	
	2	—	—	3·78	0·061
	,,	—	—	3·80	
	,,	—	—	3·83	
	,,	—	—	3·78	

In conclusion I wish to tender my sincere thanks to Dr O. Rosenheim of King's College, for many helpful criticisms and suggestions during the course of this work.

SUMMARY.

1. The benzidine method of Rosenheim and Drummond for the estimation of urinary sulphates can be carried out upon as little as 2 cc. of urine, by titrating the precipitated benzidine sulphate with 0·02 N alkali.

2. The method is also applicable to the estimation of the total sulphur in small quantities of biological material, by applying the precipitation of the benzidine sulphate to the solution of salts obtained after oxidation of the material by Benedict's method.

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XLVIII. A NEW METHOD FOR THE PREPARATION OF THE PLANT GLOBULINS.

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(*Received November 9th, 1915.*)

The plant globulins have been hitherto prepared by the extraction of the material with warm saline solutions. For this purpose sodium chloride has been most frequently employed, and relatively large volumes of solution have been necessary to obtain a satisfactory yield. In the ordinary process, furthermore, several tedious filtrations must be performed, the technique of which has been described in detail by Osborne [see, especially, Osborne, 1909].

It has been shown by Schryver [1910] that the globulins are much more soluble in solutions of those salts which lower the surface tension of water than they are in sodium chloride solution, and it was thought that by the use of these the bulk of material employed in the extraction of the globulins might be very considerably reduced and the troublesome filtration processes to a large extent avoided. At the suggestion of Dr Schryver, I have endeavoured to take advantage of the properties of certain salt solutions already referred to, and have succeeded in elaborating a process by means of which relatively large amounts of plant globulins can be prepared by processes, the chief of which can be completed within the course of a single day. The method is illustrated by the following examples.

Preparation of edestin from hemp-seed.

The hemp-seed is first ground and extracted by light petroleum. It is then filtered off on to a large Buchner-funnel, washed on the funnel with more petroleum and air-dried. The husks are then removed by sifting through coarse muslin.

500 g. of the material prepared in this way are then mixed with 500 cc. of semi-normal solution of sodium benzoate (about 7 % solution). After standing for about half an hour, so much torn blotting paper is pressed into the mass as to make it appear almost dry. The whole is then wrapped round with filter-paper and placed in a Buchner press and submitted to high pressure. About 400 cc. of liquid can be expressed. The residual mass is extracted again with about 500 cc. of the benzoate solution, and after standing for a short time, more blotting paper is added (relatively, only a small amount is necessary) and the whole is again pressed in the Buchner press, a further quantity of extract amounting to about 500 cc. being thereby obtained. The amount of edestin remaining in the residue is so small that a third extraction is almost superfluous.

The combined extracts are then thrown into about 10 litres of cold water and the whole is allowed to stand over night in a cool place. The edestin separates at the bottom of the vessel; the clear liquid is decanted off, and the creamy paste at the bottom is centrifuged. The edestin separates as a compact powder, and is then washed on the centrifuge once or twice with water, once with 95 % alcohol, once with absolute alcohol, and twice with dry ether. It is then rapidly transferred to a vacuum desiccator. A product is thereby obtained which is almost completely soluble in 10 % sodium chloride solution from which it can be obtained in a crystalline form in the usual manner. Any small impurity which is insoluble in the sodium chloride solution can be separated by centrifugalisation. 130 g. of the crude edestin (vacuum-dried) were obtained from 450 g. of the sifted meal. Semi-normal sodium salicylate solution was also tried instead of the benzoate. A violet coloured product is in this case generally obtained, owing to the action of the salicylate on the metal of the press. This colour is retained in an obstinate manner by the globulin even after recrystallisation.

Preparation of excelsin from Brazil-nuts.

The shelled nuts were passed through a hand mincing machine, and the fat was removed by extraction with light petroleum. 250 g. of fat-free powder were extracted with 250 cc. of semi-normal solution of sodium benzoate, the mass was then mixed with blotting paper, and the liquid expressed in the same manner as that described in the case of edestin. About 145 cc. of extract were obtained. The residue was then extracted twice more, each time with 250 cc., by benzoate solution (with addition of the blotting paper etc.), each extraction yielding after pressure about 250 cc. of

liquid. The combined extracts were then thrown into 10 litres of water, and the excelsin was separated and treated by the method described in the case of edestin (centrifugalisation, washing, etc.). A yield of about 20 % of the sifted meal was obtained. In another experiment the crude excelsin was dissolved when still moist after washing with water on the centrifuge, in a 6 % solution of ammonium sulphate, and this solution was then centrifuged for about 10 minutes to get rid of small amounts of insoluble matter. After 3 days' dialysis, excelsin separated from the solution, and was centrifuged off, and washed successively with 95 % alcohol, absolute alcohol, and ether, and then vacuum dried. A perfectly white powder was obtained. Yield from 250 g. of meal about 30 g.

By a similar process, a mixture of legumin and vicillin was obtained from horse-beans, and these were separated by the method described by Osborne.

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XLIX. THROMBIN AND CALCIUM CHLORIDE IN RELATION TO COAGULATION.

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(*Received September 24th, 1915.*)

The investigation which forms the subject of this article was undertaken with the object of studying more fully than has hitherto been done the quantitative relation between the time of coagulation of fibrinogen, on the one hand, and the amount of thrombokinase taking part in the process of coagulation, in presence of calcium chloride, on the other hand.

This aspect of blood coagulation has received the attention of several investigators during the last two decades. Thus E. Fuld [1902], using the plasma of geese and turkeys as source of fibrinogen, and as ferment solution the freshly prepared extract, in 0·8 % NaCl solution, of muscle, found that the equation

$$\log \frac{x}{x_0} = 0{\cdot}585 \log \frac{y}{y_0}$$

is valid when $y =$ the amount of ferment corresponding to coagulation time x and y_0 similarly corresponds to x_0.

C. J. Martin [1905], employing oxalated plasma of the sheep and dog, found that the coagulation time multiplied by the quantity of ferment (snake venom) was constant, but this law failed when a relatively large amount of venom was added, the observed times being then longer than the calculated periods. L. J. Rettger [1909] observed that the product of the coagulation time and the amount of thrombin employed was constant if the amount of fibrinogen used was fixed, but that if excess of thrombin was used a maximum amount was reached beyond which further increase no longer diminished the time of coagulation; the latter represented the time at which the contents

[1] Towards the expenses of this research a grant was made by the British Medical Association.

of the tube were sufficiently coagulated for the tube to be inverted, without the contents being spilt.

It early became obvious in the course of my investigation that the fowl's plasma employed in the experiments contained some thrombokinase, for although, when oxalated, it remained free from coagulation (a varying amount of white precipitate made its appearance on keeping) during the period it was kept under observation (four weeks at 32° C.), showing that it did not contain thrombin in appreciable quantity, nevertheless on the addition of calcium chloride coagulation always occurred after a shorter or longer period of time. It was also clear that the amount of thrombokinase present in different samples of fowl's plasma exhibited great variation. Thus coagulation at 35° C. sometimes occurred at the end of thirty minutes after the addition of calcium chloride, while when separation of plasma had been more successful its occurrence under the same conditions was not observed until the expiration of several hours. This separation was accomplished by collecting blood from the femoral artery in paraffined tubes by a paraffined cannula and obtaining plasma therefrom by centrifugalisation. Similar variations in respect of spontaneous coagulability were observed in fibrinogen solutions prepared from such samples of plasma by dilution with twenty parts of distilled water through which a stream of CO_2 had been passed. The resulting precipitate of fibrinogen was collected by centrifugalisation, dissolved in 0·85 % NaCl solution and again centrifugalised, the bulk of the resulting solution being made equal to that of the plasma, from which the fibrinogen was obtained. It was therefore clear that in estimating the coagulative effect of a given amount of thrombokinase it would be necessary to ascertain, if possible, the quantity already present in the fibrinogen solution or plasma employed. The mode in which this is effected will now be described.

In Table I a series of experiments is shown in which varying amounts of a solution of thrombokinase prepared from the testis of the rabbit were added to 0·325 cc. of fibrinogen solution in the presence of 0·05 cc. of N/10 $CaCl_2$ solution, the total volume of fluid being in each case made up to 0·5 cc. and the time of coagulation noted. The solution of thrombokinase was prepared by grinding (in a mortar) the body of the testis with sand in presence of distilled water, the resulting emulsion being made up to the original volume of the testis or to any desired multiple of the same. The extract so obtained was centrifugalised in order to remove all coarse suspended particles.

In attempting to discover if any relation exists between the time of coagulation, x (i.e. the period required for *complete* separation of fibrin),

and the amount of thrombokinase, y, added it will be observed that the product xy does not vary greatly for the last four experiments. If this relation is assumed to hold good throughout the series, then the amount of thrombokinase present in the 0·325 cc. of fibrinogen solution employed in each experiment can be shown by ordinary algebraical methods, as will be indicated for the experiments given on p. 517, to be equivalent to about 0·0068 cc. of the thrombokinase solution employed; the quantities given in the third column of Table I must therefore be increased by this amount in order that the total quantity of thrombokinase present in each experiment

TABLE I.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen solutions in presence of $CaCl_2$, a solution of NaCl (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	Fibrinogen solution (cc.)	Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	N/10 $CaCl_2$ solution (cc.)	Time of coagulation (35°) (minutes)
1	0·325	—	0·050	408
2	,,	0·097	,,	88
3	,,	0·292	,,	30
4	,,	0·877	,,	11
5	,,	2·633	,,	3

* In Experiments 3, 4 and 5 more concentrated solutions of thrombokinase were employed, in order that the total volume of fluid in each tube might not exceed 0·5 cc.

may be exhibited. This correction is made in Table II and the corrected product xy determined. For this the value 9·22 was ultimately selected and it then became possible to recalculate the times of coagulation corresponding to different amounts of thrombokinase from the equation

$$xy = \text{const.} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (1),$$

the value of the constant being taken at 9·22. This has been done in the last column of Table II and it will be seen that the observed and calculated coagulation times, except in the case of the first experiment, are in good accord; the calculated curve (the one farthest from the axes of coordinates) is given at *A*, Fig. 1. The variation observed in the first experiment will come under consideration later, when some of the factors concerned in producing such variations are taken into account.

A further series of experiments was performed, using 0·160 cc. of the same fibrinogen solution, instead of 0·325 cc. These experiments are recorded in

Table III, the product xy being given in the fifth column, after correction for the amount of thrombokinase present in the smaller amount of fibrinogen solution employed, the latter being equivalent to about 0·00335 cc. of the thrombokinase solution used. Here again the product xy does not differ very greatly in the different experiments. The value selected to represent this product is slightly greater than the mean of the observed values. By the aid of this constant the values of x corresponding to varying amounts of thrombokinase (y) are calculated in the last column of the table (curve *B*, Fig. 1).

TABLE II.

The data in the second and third columns are taken from Table I.

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
1	—	408	0·0068	2·77	9·22	1355
2	0·097	88	0·1038	9·23	,,	89
3	0·292	30	0·2988	8·96	,,	31
4	0·877	11	0·8858	9·74	,,	10·5
5	2·633	3	2·6398	7·92	,,	3·5
				mean 7·92		

TABLE III.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·160 *cc.*) *solutions in presence of* $CaCl_2$ (0·050 *cc. of* $N/10$ *solution*), *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
6	—	1050	0·00335	3·52	4·54	1355
7	0·048	88	0·05135	4·52	,,	89
8	0·144	30	0·14735	4·42	,,	31
9	0·432	11	0·43535	4·79	,,	10·5
10	1·296	3	1·29935	3·90	,,	3·5
				mean 4·23		

* In Experiments 9 and 10 more concentrated solutions of thrombokinase were employed, in order that the total volume of fluid in each tube might not exceed 0·5 cc.

Yet another series of experiments was made, a still smaller quantity of the fibrinogen solution, namely 0·050 cc., being used for each experiment (Table IV). As before a correction is made for the amount of thrombokinase

which is present in the fibrinogen solution employed. The resulting values of xy are given in the fifth column of the table. The values of x in the last column are calculated from the constant 1·42, which is somewhat higher than the mean observed value of xy. With the aid of this constant the corresponding curve C shown in Fig. 1 is constructed. It will be noted that

TABLE IV.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·050 *cc.*) *solutions in presence of* $CaCl_2$ (0·050 *cc. of* $N/10$ *solution*), *a solution of* $NaCl$ (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
11	—	1050	0·00105	1·10	1·42	1355
12	0·015	41	0·01605	0·66	,,	89
13	0·045	22	0·04605	1·01	,,	31
14	0·135	11	0·13605	1·50	,,	10·5
15	0·410	3	0·41105	1·23	,,	3·5
				mean 1·10		

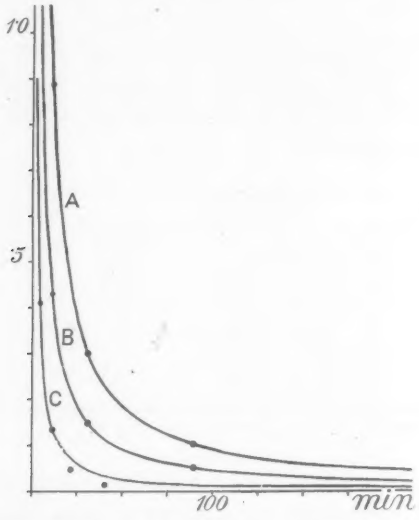

Fig. 1. Curves for the experiments given in Tables I–IV. Abscissae, time in minutes; ordinates, cc. of thrombokinase solution. C corresponds to experiments in which 0·05 cc. of fibrinogen solution was employed; B to 0·160 cc. and A to 0·325 cc.

in Exps. 11, 12 and 13 the observed are lower than the calculated values; the significance of these variations will be considered later.

All the above series of experiments were made in immediate succession, the same solutions of fibrinogen and thrombokinase being used throughout.

When the mean observed values of $x \times y$ (the latter being corrected for the amount of thrombokinase present in the fibrinogen solution used) were compared, it was found that they were approximately in the ratio of the amounts of fibrinogen solution employed, namely 0·325 cc., 0·160 cc. and 0·050 cc. The values of xy chosen in the above tables are accurately in this ratio and, as the values of x calculated therefrom are in fair agreement with the observed values, it follows that the relation between these quantities is very closely expressed by the equation

$$xy = nz \quad \text{.....(2),}$$

where x represents the time of coagulation, y and z showing the respective amounts of the solutions of thrombokinase and of fibrinogen present in the total volume of 0·5 cc. of fluid employed in each experiment, and n being a constant. To each tube 0·05 cc. of N/10 $CaCl_2$ solution was added; the temperature of experiment was 35°; the value of n is 28·4.

TABLE V.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·050 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	Fibrinogen solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	N/10 $CaCl_2$ solution (cc.)	x Time of coagulation (35°) (minutes)
11	0·050	—	0·050	46
12	,,	0·040	,,	33
13	,,	0·100	,,	25
14	,,	0·300	,,	13
15	,,	1·000	,,	8

* In Exp. 15 a more concentrated solution of thrombokinase was employed, in order that the total volume of fluid in each tube might not exceed 0·5 cc.

In the above experiments a solution of fowl's fibrinogen was employed, which contained exceedingly little thrombokinase. In the experiments recorded in Table V a relatively large amount of thrombokinase was already present in the fibrinogen solution used, coagulation (at 35°) occurring, after

the addition of calcium chloride alone, within a period of one hour. In these experiments if y (amount of thrombokinase added) is uncorrected, the product xy increases with each member of the series. The amount, e, of thrombokinase originally present in the quantity (0·050 cc.) of fibrinogen solution introduced into each tube (the total volume of fluid present being as before in each case 0·5 cc.) is determined, in terms of the equivalent amount of the thrombokinase solution employed, from the data given by the first two experiments, by putting the product xe in the former equal to the product $x'(y' + e)$ in the latter. Thus

$$xe = x'(y' + e) \quad \text{.....(3),}$$

whence

$$e = \frac{x'y'}{x - x'} \quad \text{.....(4).}$$

In this way the following series of values of e is obtained:

Exps.	11	and 12	(Table V)	$e = 0{\cdot}10$,
,,	,,	13	,,	$e = 0{\cdot}12$,
,,	,,	14	,,	$e = 0{\cdot}12$,
,,	,,	15	,,	$e = 0{\cdot}19$,
				mean 0·13.

The value of e finally chosen was 0·136.

If now to the amounts of thrombokinase introduced into each tube the equivalent (in terms of the thrombokinase solution employed) amount of thrombokinase originally present in the quantity (0·050 cc.) of fibrinogen

TABLE VI.

Recalculation of values of y (thrombokinase) and x (time of coagulation) from Table V.

No. of experiment	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Corresponding value of $x_{calculated}$ (minutes)	x_{found} (minutes)
11	0·136	6·25	6·0	44	46
12	0·176	5·80	,,	34	33
13	0·236	5·90	,,	25	25
14	0·436	5·66	,,	14	13
15	1·136	9·09	,,	5	8
		mean 6·54			

solution used is added, the corrected values of y are obtained and from these the true values of the product xy are found. These are set forth in Table VI. The observed values of x, corresponding to the corrected values of y, are shown in Fig. 2, curve A being calculated for $xy = 6$, this being the value

ultimately selected. It will be noted that the observed and calculated values agree fairly closely. This series of experiments illustrates how a relatively heavily contaminated solution of fibrinogen—and the same applies to the plasma from which it is obtained—may be employed for the purpose of investigation, provided the amount of admixture of thrombokinase is determined. The disadvantage of such solutions, however, lies in the circumstance that the range of investigation is limited because the time of coagulation for very small concentrations of thrombokinase, corresponding to the lower

TABLE VII.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·160 cc. for Exps. 6 to 10; 0·325 cc. for Exps. 1 to 5) solutions in presence of $CaCl_2$ (0·050 cc. of N/10 solution), a solution of NaCl (0·85 %) being added in amount sufficient to make a total volume of 0·5 cc. in each experiment. These experiments complete the series, of which those recorded in Table V form a part.

No. of experiment	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000* (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x, corresponding to $y_{corrected}$ (minutes)
1	—	52	0·884	45·90	39·0	44
2	0·030	40	0·914	36·56	,,	43
3	0·100	31	0·984	30·52	,,	40
4	0·300	28	1·184	23·14	,,	33
5	1·000	20	1·884	37·68	,,	21
				mean 34·76		
6	—	54	0·436	23·52	19·2	44
7	0·035	38	0·471	17·90	,,	41
8	0·100	30	0·536	16·08	,,	36
9	0·300	27	0·736	19·87	,,	26
10	1·000	14	1·436	20·08	,,	13
				mean 19·49		

* In Exps. 4, 5, 9 and 10 more concentrated solutions of thrombokinase were employed.

parts of the curves shown in the figures (e.g. to the right of the vertical lines at 44 and 27 in Figs. 2 and 3 respectively on the axis of abscissae), cannot be determined, since these represent amounts of thrombokinase which are less than that already present in the plasma or fibrinogen solution employed.

For the sake of comparison with Table VI the remaining experiments of the group from which Table V is constructed are given in Table VII, the corresponding curves (*B* and *C*) being shown in Fig. 2. As in the preceding series the observed values of x do not differ greatly from those

calculated with the aid of the values of xy chosen (39·0, 19·2 and 6·0), which it will be noted are proportional to the amounts of fibrinogen solution (0·325 cc., 0·160 cc. and 0·050 cc. respectively) employed; thus conforming to equation (2).

The enquiry now presents itself, Does the time of coagulation represent the time required to convert prothrombin into thrombin or does it correspond to the period during which thrombin acts upon fibrinogen, giving rise thereby to the appearance of fibrin or does each of these processes occupy an

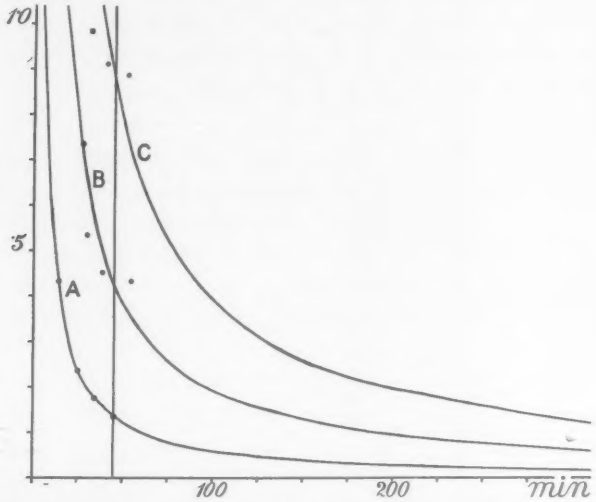

Fig. 2. Curves for the experiments given in Tables V–VII. Abscissae, time in minutes; ordinates, cc. of thrombokinase solution. The vertical line at 44 on the axis of abscissae represents the outer limit of investigation of the curves. Curve *A* corresponds to experiments in which 0·050 cc. of fibrinogen solution was employed; *B* to 0·160 cc. and *C* to 0·325 cc.

appreciable fraction of the coagulation time? To determine this point thrombin in the form of venom from *Echis carinatus* [Barratt 1913, 1] was added to fibrinogen in varying amounts, and the period of coagulation (at 35°) noted. These experiments, which are given in Table VIII, fall into two groups: in the first (Exps. 1 to 14) plasma to which calcium chloride had been added, as in the preceding series, was employed; in the second group (Exps. 15 to 19) oxalated plasma was used. It will be observed that the course of the first group is similar to that of the experiments already recorded, in which thrombokinase was used and, as in the latter, the times

of coagulation have been calculated on the assumption that the product xy is constant for each of the three amounts of fibrinogen employed, the observed and calculated values being in fair agreement, except for the control experiments 1, 6 and 11, in which the observed periods were longer than the

TABLE VIII.

Determination of the coagulation time of a series of mixtures of venom of Echis carinatus and calcified fowl's plasma (0·325 *cc. for Exps.* 1 *to* 5; 0·160 *cc. for Exps.* 6 *to* 10 *and* 0·050 *cc. for Exps.* 11 *to* 14, *in presence of* 0·050 *cc. of* $N/10$ $CaCl_2$ *solution*) *or oxalated fowl's plasma* (0·325 *cc. for Exps.* 15 *to* 19), *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	y Venom solution, 1 in 300,000* (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	$x \times y_{corrected}$	Value of xy selected	Calculated values of x (minutes)	Plasma (cc.)
1	—	52	0·11	5·7	3·00	27	0·325 calcified
2	0·030	20	0·14	2·8	,,	21	,, ,,
3	0·100	14	0·21	3·2	,,	14	,, ,,
4	0·300	4	0·41	1·6	,,	7	,, ,,
5	1·000	2	1·11	2·2	,,	3	,, ,,
6	—	54	0·054	2·9	1·476	27	0·160 ,,
7	0·035	14	0·089	1·2	,,	17	,, ,,
8	0·100	10	0·154	1·5	,,	10	,, ,,
9	0·300	4	0·354	1·4	,,	4	,, ,,
10	1·000	2	0·737†	1·5	,,	2	,, ,,
11	—	44	0·0169	0·74	0·461	27	0·050 ,,
12	0·040	10	0·0469	0·47	,,	10	,, ,,
13	0·100	4	0·1169	0·47	,,	4	,, ,,
14	0·300	2	0·2310†	0·46	,,	2	,, ,,
15‡	—	∞	—	—	3·44	∞	0·325 oxalated
16	0·030	129	—	3·87	,,	115	,, ,,
17	0·100	33	—	3·30	,,	34	,, ,,
18	0·300	9	—	2·70	,,	11	,, ,,
19	0·590	6	—	3·54	,,	6	,, ,,
				mean 3·97			

* In Exps. 4, 5, 9, 10, 14, 18 and 19 more concentrated solutions of venom were employed.
† Effective values of y (cp. p. 526).
‡ In Exps. 15 to 19 the plasma employed was not the same as that used for Exps. 1 to 14.

calculated. In control experiments of this type irregular variation from the calculated periods is met with more frequently than in experiments in which thrombokinase or thrombin has been added. If the experiments are repeated such differences do not appear to be constant, as may be seen by comparing the corresponding experiments in the preceding tables. The degree of

contamination of the plasma used can be determined as in the previous experiments, except that the estimation is now made in terms of thrombin instead of thrombokinase. Finally it will be noted that the values of xy selected (3·00, 1·476 and 0·461 respectively) are in the same ratio as the amounts of fibrinogen solution added (0·325 cc. in Exps. 1 to 5; 0·160 cc. in Exps. 6 to 10; 0·050 cc. in Exps. 11 to 14), equation (2) being valid, within the limits of experimental error, for these experiments with thrombin. In the second series of experiments shown in Table VIII (Exps. 15 to 19), in which the action of thrombin takes place in presence of potassium oxalate (a solution of fibrinogen different from that employed in the preceding experiments being used), the product xy is, as in the previous experiments, constant and the calculated values of x exhibit a good agreement with the observed values; in these experiments no correction for contamination of plasma is required, the plasma remaining uncoagulated when kept in presence of potassium oxalate. The observed coagulation times are reproduced in Fig. 3; the curves are calculated, *A* representing Exps. 1–5; *B* 6–10 and *C* 11–14.

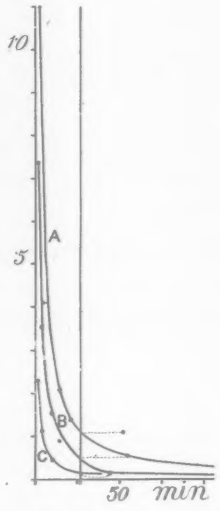

Fig. 3. Curves for the experiments given in Table VIII. Abscissae, time in minutes; ordinates, cc. of 1 in 300,000 venom solution. The vertical line at 27 on the axis of abscissae represents the outer limit of investigation of the curves. Curve *C* corresponds to experiments in which 0·050 cc. of fibrinogen solution was employed, curve *B* to 0·160 cc. and curve *A* to 0·325 cc.

The above experiments, which, like the preceding experiments made with thrombokinase, take place according to equation (2), indicate that the time of coagulation is essentially the period required for the conversion of fibrinogen into fibrin.

Since the experiments with thrombin in Table VIII indicate therefore that the influence of thrombin (y) upon the coagulation time (x) in presence of a given amount of fibrinogen (z) takes place according to equation (2)

$$xy = nz$$

(n in this particular series of experiments being 10·6), and since, in addition, this equation also expresses the action of thrombokinase in causing coagulation

(Tables I to VII), it follows that thrombokinase and thrombin are interchangeable terms, in respect of fibrin production, in other words that a given amount of thrombokinase, in presence of calcium chloride, will produce a fixed amount of thrombin, whatever may be the concentration of prothrombin, provided of course that the latter is present in excess. As soon as this amount of thrombin has been produced the thrombokinase added has either disappeared or has become so far modified as to be incapable of further action in the direction of giving rise to the production of thrombin in presence of prothrombin and calcium chloride. In no other way can the similarity of the results obtained with thrombin (Table VIII) to those obtained with thrombokinase (Tables I to IV and V to VII) be explained.

The validity of equation (2) may be tested in a manner which in some respects offers an advantage as regards the ease with which simultaneous observations may be made. It is obvious that if, in the equation

$$xy = nz,$$

y is made proportional to z, i.e.

$$y = dz,$$

where d is a constant, then the equation becomes

$$xdz = nz,$$

or

$$x = \frac{n}{d} = \text{const.} \quad \text{.....(5),}$$

that is to say the coagulation time is independent of the concentration of fibrinogen present in solution. This condition is realised if thrombokinase is first added to fibrinogen solution and the mixture thus obtained introduced in varying amounts into tubes, each containing the same quantity of decinormal calcium chloride solution, the total volume of fluid in each case being made up to 0·5 cc. This was in fact done in the series of experiments recorded in Tables I to IV. Thus in the three experiments numbered 2, 7 and 12 a suitable admixture of thrombokinase and fibrinogen solutions was first made and from this amounts of 0·422 cc., 0·208 cc. and 0·065 cc. (containing respectively 0·325 cc., 0·160 cc. and 0·050 cc. of fibrinogen solution) were transferred to tubes into which the requisite amounts of N/10 $CaCl_2$ solution and 0·85 % NaCl solution had already been introduced; and so on with the remaining tubes with the exception of those numbered 1, 6 and 11, to which no thrombokinase was added.

It will be observed that corresponding tubes exhibit equi-coagulative periods, except in Exps. 12 and 13, Table IV; some of the causes of such variations are considered on p. 540.

The influence of calcium chloride upon the action of thrombokinase and thrombin in effecting coagulation of a fibrinogen solution.

The constant n in equation (2) (p. 516) is, in the mathematical sense, a function of all those potential variables which for these experiments are kept constant. Of the latter two are more immediately important, namely the concentration of $CaCl_2$ and the temperature, the former of which alone will be considered here.

To this end a series of forty experiments with thrombokinase, similar in type to those shown in Tables I and II, was carried out in the numerical order given in Tables IX and X. These experiments fall into two groups: in the first (Table IX) 0·325 cc. of fibrinogen solution was employed in each experiment; in the second (Table X) 0·160 cc. of fibrinogen solution was used.

TABLE IX.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·325 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment	N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
1	0·075	0·1	44	0·148	7·98	54·0
3	,,	0·2	31	0·248	,,	32·2
5	,,	0·3	23	0·348	,,	22·9
7	,,	0·6	13	0·648	,,	12·2
9	,,	1·0	10	1·048 [0·897]	,,	7·6 [8·9]
11	0·125	0·1	50	0·148	9·42	63·6
13	,,	0·2	39	0·248	,,	38·0
15	,,	0·3	23	0·348	,,	27·1
17	,,	0·6	13	0·648	,,	14·5
19	,,	1·0	10	1·048 [0·897]	,,	9·0 [10·5]
21	0·5	0·1	300	0·148	42·8	289·5
23	,,	0·2	129	0·248	,,	173·0
25	,,	0·3	100	0·348	,,	123·2
27	,,	0·6	59	0·648	,,	66·0
29	,,	1·0	46	1·048 [0·897]	,,	40·8 [47·8]
31	1·5	0·1	>6500	0·148	327·5	2218
33	,,	0·2	>5000	0·248	,,	1323
35	,,	0·3	>4000	0·348	,,	944
37	,,	0·6	1200	0·648	,,	505
39	,,	1·0	780	1·048 [0·897]	,,	313 [365]

In these two tables the experiments are seen to be arranged in four groups, each of five experiments, the amounts of decinormal $CaCl_2$ solution employed in each group being respectively 0·075 cc., 0·125 cc., 0·5 cc. and 1·5 cc. The experiments in each individual group are arranged, as in the preceding tables, in the order of the amount of thrombokinase introduced into each tube. As in the preceding experiments each tube contained 0·5 cc. of fluid. When the amount of thrombokinase or calcium chloride solution was so great that this amount would have been exceeded, more concentrated solutions were employed, but in the tables, for the sake of uniformity and consequent ease of calculation the amount used is always referred to the same standard of concentration.

As before the first step in dealing with these experiments was to determine, by means of the method given on p. 517, the amount of thrombokinase contained in the fibrinogen solution used and, at the same time, to ascertain if for each group values of xy could be obtained which would satisfy equation (1) (p. 513), namely

$$xy = \text{const.}$$

The amount of thrombokinase present in 0·325 cc. and 0·160 cc. of the fibrinogen solution employed was estimated to be equal to 0·048 cc. and 0·024 cc. respectively of the thrombokinase solution used. When these quantities were added to the thrombokinase employed, the total amount of thrombokinase taking part in each experiment became known and it was possible to select values representing the product of x (coagulation time) and y (amount of thrombokinase present) for each group. The values of xy so chosen yield, it will be seen, calculated values of x differing (except in cases to which reference will shortly be made) from the observed values only within the limits of experimental error.

It will further be seen that the values of xy (7·98 and 3·61 respectively) selected for the first group in Tables IX and X, in which the amount of $CaCl_2$ solution employed is lowest, are approximately in the ratio of the amounts of fibrinogen solution added (0·325 cc. and 0·160 cc. respectively), but that with increasing amounts of $CaCl_2$ a departure from this ratio is observed.

It has already been pointed out that certain calculated values of x differ from those actually observed, to an extent which lies beyond the range of experimental error. These fall into two groups: (1) those in which a very large amount of thrombokinase has been employed (Exps. 9, 19, 29 and 39, Table IX, and 8, 10, 18, 20, 28, 30, 38 and 40, Table X); and (2) those in

which a very large amount of $CaCl_2$ has been used (Exps. 31 to 40). The latter group will be dealt with later but the former may now conveniently be considered. For a long time this class of variations from the calculated value of the coagulation time (x) presented a difficulty, until it was observed that the coagulation time became stationary as soon as the amount of

TABLE X.

Determination of the coagulation time of a series of mixtures of thrombokinase and fibrinogen (0·160 cc.) solutions in presence of $CaCl_2$, a solution of NaCl (0·85 %) being added in amount sufficient to make a total volume of 0·5 cc. in each experiment.

No. of experiment	N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
2	0·075	0·1	30	0·124	3·61	29·1
4	,,	0·2	20	0·224	,,	16·1
6	,,	0·3	13	0·324	,,	11·1
8	,,	0·6	10	0·624 [0·442]	,,	5·8 [8·2]
10	,,	1·0	10	1·024 [0·442]	,,	3·5 [8·2]
12	0·125	0·1	39	0·124	3·73	30·1
14	,,	0·2	17	0·224	,,	16·7
16	,,	0·3	11	0·324	,,	11·5
18	,,	0·6	7	0·624 [0·442]	,,	6·0 [8·5]
20	,,	1·0	7	1·024 [0·442]	,,	3·6 [8·5]
22	0·5	0·1	56	0·124	6·59	53·2
24	,,	0·2	26	0·224	,,	29·4
26	,,	0·3	20	0·324	,,	20·4
28	,,	0·6	15	0·624 [0·442]	,,	10·6 [14·9]
30	,,	1·0	12	1·024 [0·442]	,,	6·4 [14·9]
32	1·5	0·1	>6500	0·124	31·03	250·0
34	,,	0·2	1440	0·224	,,	138·7
36	,,	0·3	780	0·324	,,	95·7
38	,,	0·6	780	0·624 [0·442]	,,	49·7 [70·2]
40	,,	1·0	780	1·024 [0·442]	,,	30·3 [70·2]

thrombokinase (y) reached a certain value, beyond which further increase caused no appreciable shortening of the time of coagulation. Whatever may be the mode, physical or physico-chemical, in which this event takes place, its occurrence may be conveniently expressed by saying that in practice the effective maximum value of thrombokinase is fixed and cannot be exceeded. This value was determined approximately by substituting varying lower values of y for the highest amounts actually employed and selecting those

which gave the best concordance between the calculated and observed values of x[1]. Since the maximum effective values of y which were at first selected for the two series of experiments (Tables IX and X) were found to be approximately in the ratio of the amounts of fibrinogen solution employed, it was assumed that this relation actually existed and on this assumption the values finally adopted were chosen (0·897 cc. of thrombokinase solution when

TABLE XI.

The experiments recorded in Table IX, rearranged in a form suitable for calculating the values of a and b in the equation $w^2 = ax - b$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	$a=\frac{w_1^2-w^2}{x_1-x}$	$b=ax-w^2$
9	0·075	0·897	[10] 8·9		0·0504
19	0·125	,,	[10] 10·5	0·0062	,,
29	0·5	,,	[46] 47·8	0·0063	,,
39	1·5	,,	[780] 365·0	,,	,,
7	0·075	0·648	[13] 12·2		,,
17	0·125	,,	[13] 14·5	0·00455	,,
27	0·5	,,	[59] 66·0	,,	,,
37	1·5	,,	[1200] 505·0	,,	,,
5	0·075	0·348	[23] 22·9		,,
15	0·125	,,	[23] 27·1	0·00246	,,
25	0·5	,,	[100] 123·2	,,	,,
35	1·5	,,	[>4000] 944·0	,,	,,
3	0·075	0·248	[31] 32·2		,,
13	0·125	,,	[39] 38·0	0·00174	,,
23	0·5	,,	[129] 173·0	,,	,,
33	1·5	,,	[>5000] 1323·0	,,	,,
1	0·075	0·148	[44] 54·0		,,
11	0·125	,,	[50] 63·6	0·00103	,,
21	0·5	,,	[300] 289·5	,,	,,
31	1·5	,,	[>6500] 2218·0	,,	,,

0·325 cc. of fibrinogen solution was employed and 0·442 cc. of the former for 0·160 cc. of the latter). With the aid of these values of y and of the values of the product xy already selected the divergent values of x under consideration were recalculated; these are given within brackets in the last column of the tables and are seen to approach more nearly to the observed coagulation times than do the lower values obtained when the full amounts of thrombokinase present are taken.

[1] In this way the effective values of y in Table VIII were obtained.

In order to study the effect of $CaCl_2$ upon the time of coagulation the experiments given in Tables IX and X were rearranged in Tables XI and XII respectively according to the effective amounts of thrombokinase present. This course was adopted because it was found, when searching for a relationship between the amounts of $CaCl_2$ (w) present and the calculated coagulation times (x), in presence of a fixed amount of thrombokinase (y), that the coagulation times, when plotted as abscissae with the corresponding amounts

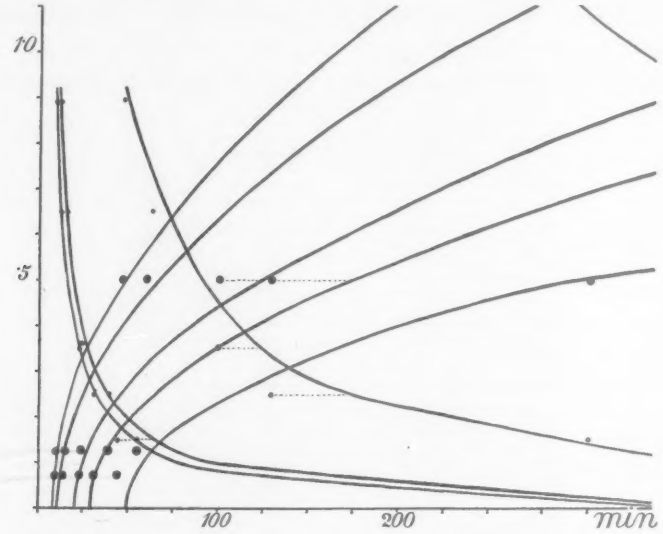

Fig. 4. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables IX and XI. Abscissae (x), time in minutes; ordinates, cc. of solution of thrombokinase (y) for hyperbolic curves (·) or of N/10 calcium chloride (w) for parabolic curves (•). The amount of fibrinogen solution employed in each experiment is 0·325 cc. The hyperbolic curve nearest to the axes corresponds to those experiments in which 0·075 cc. of decinormal calcium chloride solution was employed; the remaining hyperbolic curves to 0·125 cc., 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0·897 cc. of thrombokinase solution was employed; the remaining parabolic curves to 0·648 cc., 0·348 cc., 0·248 cc. and 0·148 cc. respectively.

of decinormal $CaCl_2$ solution as ordinates, lay on a curve which appeared to be parabolic (Fig. 4 for Tables IX and XI; Fig. 5 for Tables X and XII). If parabolic the values of x must satisfy the equation

$$w^2 = ax - b \quad \text{.....(6),}$$

where a and b are constants. It will be observed that in each table five curves are investigated, corresponding to five different concentrations of

thrombokinase and that four points on each curve are determined. If only a single curve had been studied a larger number of points could have been determined, but the advantage of investigating simultaneously a series of values of w would have been lost.

The manner in which a and b were determined is sufficiently indicated in the 5th and 6th columns of Tables XI and XII. Guidance in selecting

TABLE XII.

The experiments recorded in Table X, rearranged in a form suitable for calculating the values of a and b in the equation $w^2 = ax - b$. The observed coagulation times are given within brackets in the fourth column.

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Thrombokinase solution (testis of rabbit) diluted 1 in 1000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	$a=\frac{w_1^2-w^2}{x_1-x}$	$b=ax-w^2$
10	0·075	0·442	[10] 8·2	0·0362	0·290
20	0·125	,,	[7] 8·5		,,
30	0·5	,,	[12] 14·9	,,	0·291
40	1·5	,,	[780] 70·2	,,	0·290
8	0·075	,,	[10] 8·2	0·0362	,,
18	0·125	,,	[7] 8·5		,,
28	0·5	,,	[15] 14·9	,,	,,
38	1·5	,,	[780] 70·3	,,	,,
6	0·075	0·324	[13] 11·1	0·0265	,,
16	0·125	,,	[11] 11·5		,,
26	0·5	,,	[20] 20·3	,,	,,
36	1·5	,,	[780] 95·7	,,	,,
4	0·075	0·224	[20] 16·1	0·01835	,,
14	0·125	,,	[17] 16·7		,,
24	0·5	,,	[26] 29·4	,,	,,
34	1·5	,,	[1440] 138·7	,,	,,
2	0·075	0·124	[30] 29·1	0·01015	,,
12	0·125	,,	[39] 30·1		,,
22	0·5	,,	[56] 53·2	,,	,,
32	1·5	,,	[>6500] 250·0	,,	,,

final values for these constants is afforded by studying the effect, upon the recalculated values of x, produced by slightly varying the values first selected. When preliminary determinations of a and b had been made it was found that the values of a for each curve were roughly proportional to the amounts of thrombokinase employed, while the values of b were not far different for all the curves. It was therefore concluded that these indications were valid and values of a and b were accordingly selected which appeared best to fulfil the two conditions thus foreshadowed. It will be noted that, with the

values selected, the calculated coagulation times agree with those actually found, within the limits of experimental error for the first three experiments of each group, but that a divergence between the two occurs when the equivalent of 1·5 cc. of decinormal $CaCl_2$ solution is employed (Exps. 39, 37, 35, 33 and 31, Table XI; Exps. 40, 38, 36, 34 and 32, Table XII) and that the divergence is more marked when 0·160 cc. of fibrinogen solution is employed (Table XII) than when 0·325 cc. is used (Table XI). In other words equation (6) fails when the concentration of $CaCl_2$ is increased relatively to the fibrinogen solution employed to such an extent that the ratio of the

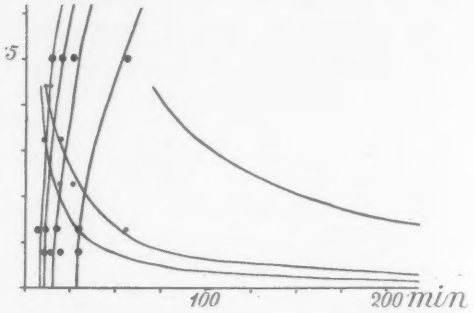

Fig. 5. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables X and XII. Abscissae (x), time in minutes; ordinates, cc. of solution of thrombokinase (y) for hyperbolic curves (·) and of N/10 calcium chloride (w) for parabolic curves (•). The amount of fibrinogen solution employed in each experiment is 0·160 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0·075 cc. and 0·125 cc. respectively of N/10 $CaCl_2$ were employed, the remaining hyperbolic curves to 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0·442 cc. of thrombokinase solution was employed; the remaining parabolic curves to 0·324 cc., 0·224 cc. and 0·124 cc. respectively.

two reaches 4·6 (Table XI). With this ratio the observed values of x are two to four times as great as the calculated; when the ratio becomes 9·4 (Table XII) the former are roughly ten times as great as the latter.

Since a has been found to be proportional to y, in other words since

$$a = ky,$$

where k is a constant, equation (6) may be written

$$w^2 = kxy - b \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots(7).$$

It was further observed that the values of xy when the amount of $CaCl_2$ (w) was small were approximately proportional to the amount of fibrinogen

solution (z) employed. It was therefore determined to introduce this relationship into the above equation by putting $k = p$, where p is a constant, and at the same time to express the constant b in the form of a multiple of p, namely pn, n of course being itself a constant. The equation thus becomes

$$w^2 = \frac{p}{z}xy - pn,$$

or

$$w^2 = p\left(\frac{xy}{z} - n\right) \quad \text{.....(8).}$$

If in the forty experiments at present under consideration approximate values of xy are first determined, in the manner indicated in Tables IX and X, and if from these the values of the constants p and n are next determined, it will be found that the estimated values of n lie close together for all the groups in both series of experiments, so that it seems legitimate to infer that n is independent of variations of z. This inference was adopted in calculating p and n in the experiments recorded in Tables IX to XII, the values selected being

$$n = 22{\cdot}12,$$
$$p_{0{\cdot}325} = 0{\cdot}00228,$$
$$p_{0{\cdot}160} = 0{\cdot}0131.$$

The mode of determining the two constants is illustrated in the series of experiments recorded in Tables XIII to XVI.

Having determined the values of the constants p and n from the approximate values of xy first reached according to the method indicated in Tables IX and X, the values of the product xy for varying concentrations of $CaCl_2$ may now be recalculated by means of equation (8). It thus becomes possible to determine the slight variations in the value of xy corresponding to the lower values of w, variations which may be too small to be determined satisfactorily by direct observation of coagulation times. It was in this way that the final values of xy, given in Tables IX and X were chosen, the effect of slight variations of p and n upon the calculated values of xy having been carefully studied before the values of these constants ultimately selected were adopted.

It may here be pointed out as a matter of practical convenience that the determination of p and n [equation (8)] is much simpler than the estimation of a and b [equation (6)] or of k and b [equation (7)]. As already mentioned n is independent of w, xy and z; p remains a function of z, but is independent of xy and w. If $w = 0$ in equation (8), a condition of experiment which

cannot be realised experimentally however, xy becomes proportional to z and equation (2) (p. 516) is obtained, thus

$$\frac{xy}{z} - n = 0,$$

or

$$xy = nz.$$

It is thus seen that equation (2) is theoretically admissible only if w is relatively very small. If w is not sufficiently small to be negligible, then the complete formula

$$xy = \left(\frac{w^2}{p} + n\right) z$$

must be used.

Equation (6) is investigated in Tables XI and XII with a minimal addition of 0·075 cc. of decinormal calcium chloride solution (w) in each 0·5 cc. of fluid. The equation is valid until w falls to 0·03 cc.; below this amount a gradual lengthening of the coagulation time occurs (the amount of thrombokinase added remaining, as before, constant), the observed values of x being greater than the calculated values, so that equations (6) to (8) are no longer applicable. The complete w, x curve which is of the type shown in 2, Fig. 8 (p. 539) appears to be made up of two curves: (1) the parabolic curve already described; and (2) a flat curve, lying below 0·03 on the axis of ordinates, which as it proceeds to the right approaches the axis of abscissae. The latter curve, the accurate investigation of which is difficult, will not be further considered here; it appears to represent the extent to which thrombin has been produced from prothrombin in presence of relatively low percentages of calcium chloride. The form of the complete curve shows that it is not a hyperbola, such as is obtained when the inhibitory action of amboceptor upon complement is represented [Barratt 1913, 2 and 3]; the application of the equation for a hyperbola to the complete w, x curve is impossible.

The enquiry now presents itself, whether the influence of calcium chloride upon coagulation is concerned with the activity of thrombokinase or with that of thrombin. To this end the series of experiments recorded in Tables IX to XII were repeated, with thrombin, in the form of snake venom, in place of thrombokinase. In these experiments (Tables XIII and XIV) the values of xy were first determined as in the preceding series and it is seen that equation (1)

$$xy = \text{const.}$$

is applicable just as when thrombokinase is employed, allowance being made as before for the amount of thrombokinase already present in the fibrinogen

solution employed, this being determined in terms of the thrombin solution employed. It will be observed, however, that in addition to the four groups given in each of the preceding Tables (IX and X) an additional group has been added in which no calcium chloride has been used, venom thrombin (and the same is true of thrombin produced from prothrombin by the action of thrombokinase) being capable of causing coagulation of fibrinogen when no calcium chloride has been added or when, by adding potassium oxalate,

TABLE XIII.

Determination of the coagulation time of a series of mixtures of venom and fibrinogen (0·325 *cc.*) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 *cc. in each experiment.*

No. of experiment		N/10 $CaCl_2$ solution (cc.)	y Venom (*Echis carinatus*) solution, 1 in 900,000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
I	1	—	4·5	6	4·650	24·30	5·2
	11	—	0·6	35	0·750	,,	32·4
	21	—	0·115	102	0·265	,,	91·5
	31	—	0·045	120	0·195	,,	124·0
	41	—	0·015	152	0·165	,,	147·0
	51	—	—	510	0·150	,,	162·0
II	3	0·075	4·5	4	2·94 [4·604]	11·12	3·8
	13	,,	0·6	12	0·704	,,	15·8
	23	,,	0·035	50	0·209	,,	53·2
	33	,,	0·015	76	0·149	,,	74·7
	43	,,	0·005	122	0·119	,,	93·5
	53	,,	—	160	0·104	,,	107·0
III	5	0·125	4·5	4	2·94 [4·604]	11·25	3·8
	15	,,	0·6	12	0·704	,,	16·6
	25	,,	0·115	42	0·209	,,	53·9
	35	,,	0·045	76	0·149	,,	75·5
	45	,,	0·015	102	0·119	,,	94·5
	55	,,	—	160	0·104	,,	108·0
IV	7	0·5	4·5	5	2·94 [4·604]	14·19	4·8
	17	,,	0·6	18	0·704	,,	20·1
	27	,,	0·115	62	0·209	,,	67·9
	37	,,	0·045	100	0·149	,,	95·1
	47	,,	0·015	137	0·119	,,	119·2
	57	,,	—	182	0·104	,,	136·2
V	9	1·5	4·5	44	2·94 [4·604]	39·10	13·3
	19	,,	0·6	94	0·704	,,	55·5
	29	,,	0·115	330	0·209	,,	187·3
	39	,,	0·045	413	0·149	,,	262·3
	49	,,	0·015	640	0·119	,,	319·5
	59	,,	—	640	0·104	,,	376·0

the calcium salt present in solution has been reduced to a point determined by the extremely low solubility of calcium oxalate. It is further to be noted that when no calcium chloride has been added the time of coagu'ation, after the addition of varying amounts of venom, is longer than is the case in presence of a small quantity of calcium chloride. For the present, however, attention will be given only to the IInd to Vth groups of Tables XIII and XIV, which alone can be compared with the preceding experiments with thrombokinase in Tables IX and X.

TABLE XIV.

Determination of the coagulation time of a series of mixtures of venom and fibrinogen (0·050 cc.) *solutions in presence of* $CaCl_2$, *a solution of NaCl* (0·85 %) *being added in amount sufficient to make a total volume of* 0·5 cc. *in each experiment.*

	No. of experiment	N/10 $CaCl_2$ solution (cc.)	y Venom (*Eckis carinatus*) solution, 1 in 900,000 (cc.)	x Time of coagulation (minutes)	$y_{corrected}$ (cc.)	Value of xy selected	$x_{calculated}$ (minutes)
I	2	—	4·5	4	4·560	7·30	1·6
	12	—	0·6	11	0·660	,,	11·0
	22	—	0·115	35	0·175	,,	41·7
	32	—	0·045	76	0·105	,,	69·6
	42	—	0·015	102	0·075	,,	97·2
	52	—	—	640	0·060	,,	122·0
II	4	0·075	4·5	4	0·453 [4·516]	1·73	3·8
	14	,,	0·6	5	0·453 [0·616]	,,	3·8
	24	,,	0·115	16	0·121	,,	14·3
	34	,,	0·045	25	0·061	,,	28·4
	44	,,	0·015	49	0·031	,,	35·8
	54	,,	—	103	0·016	,,	108·1
III	6	0·125	4·5	4	0·453 [4·516]	1·80	4·0
	16	,,	0·6	5	0·453 [0·616]	,,	4·0
	26	,,	0·115	16	0·121	,,	14·9
	36	,,	0·045	25	0·061	,,	29·5
	46	,,	0·015	55	0·031	,,	58·0
	56	,,	—	102	0·016	,,	112·4
IV	8	0·5	4·5	6	0·453 [4·516]	3·26	7·2
	18	,,	0·6	8	0·453 [0·616]	,,	7·2
	28	,,	0·115	27	0·121	,,	27·0
	38	,,	0·045	48	0·061	,,	53·5
	48	,,	0·015	93	0·031	,,	105·0
	58	,,	—	180	0·016	,,	204·0
V	10	1·5	4·5	31	0·453 [4·516]	15·75	34·8
	20	,,	0·6	40	0·453 [0·616]	,,	34·8
	30	,,	0·115	159	0·121	,,	130·1
	40	,,	0·045	248	0·061	,,	258·0
	50	,,	0·015	510	0·031	,,	507·4
	60	,,	—	>2500	0·016	,,	985·0

When calcified venom was employed the lowest values of xy first selected in the two series (11·12 and 1·73 were ultimately chosen) were found to be very nearly in the ratio of the amounts of fibrinogen solution added (0·325 cc. and 0·050 cc. respectively), indicating that these groups conformed approximately to equation (2)

$$xy = nz.$$

It was also found that the thrombin of venom was effective until an amount equivalent to 2·94 cc. of the venom solution for the first series (Table XIII) and 0·453 cc. for the second series (Table XIV) was used; increase beyond this amount did not cause a corresponding diminution of the coagulation

TABLE XV.

The experiments recorded in Table XIII rearranged in a form suitable for calculating the values of p and n in the equation $w^2 = p\left(\frac{xy}{z} - n\right)$. *The observed coagulation times are given within brackets in the fourth column.*

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Venom solution (*Echis carinatus*), 1 in 300,000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	xy	$p = \frac{w_1^2 - w^2}{\frac{x_1 y_1}{z} - \frac{xy}{z}}$	$n = \frac{xy}{z} - \frac{w^2}{p}$
3	0·075	2·94	[4] 3·8	11·12	0·026	34·01
5	0·125	,,	[4] 3·8	11·25		34·04
7	0·5	,,	[5] 4·8	14·19	,,	34·07
9	1·5	,,	[44] 13·3	39·10	,,	33·90
13	0·075	0·704	[12] 15·8	11·12		34
15	0·125	,,	[12] 16·6	11·25	,,	,,
17	0·5	,,	[18] 20·1	14·19	,,	,,
19	1·5	,,	[94] 55·5	39·10	,,	,,
23	0·075	0·209	[50] 53·2	11·12		,,
25	0·125	,,	[42] 53·9	11·25	,,	,,
27	0·5	,,	[62] 67·9	14·19	,,	,,
29	1·5	,,	[330] 187·3	39·10	,,	,,
33	0·075	0·149	[76] 74·7	11·12		,,
35	0·125	,,	[76] 75·5	11·25	,,	,,
37	0·5	,,	[100] 95·1	14·19	,,	,,
39	1·5	,,	[413] 262·3	39·10	,,	,,
43	0·075	0·119	[122] 93·5	11·12		,,
45	0·125	,,	[102] 103·2	11·25	,,	,,
47	0·5	,,	[137] 119·2	14·19	,,	,,
49	1·5	,,	[640] 319·5	39·10	,,	,,
53	0·075	0·104	[160] 107·0	11·12		,,
55	0·125	,,	[160] 108·0	11·25	,,	,,
57	0·5	,,	[182] 136·2	14·19	,,	,,
59	1·5	,,	[640] 376·0	39·10	,,	,,

time. In these respects the experiments with venom resemble those already made with thrombokinase and are in agreement with the series shown in Table VIII.

The experiments with calcified venom were now, like the series given in Tables IX and X, rearranged (Tables XV and XVI) for convenience of ascertaining whether or no equation (8)

$$w^2 = p\left(\frac{xy}{z} - n\right)$$

was applicable. It was found that (except in the case of the last group of experiments shown in Tables XIII and XIV) with very slight changes in the values of xy first chosen, values of p and n (given in Tables XV and XVI) could be selected such that the coagulation times recalculated therefrom, given in the tables, were in agreement with the observed periods, within the limits of experimental error. The curves for Tables XIII and XV are given in Fig. 6; those for Tables XIV and XVI in Fig. 7.

It thus appears that in presence of calcium chloride both series (Tables IX to XII and XIII to XVI respectively) behave alike, the same equation being applicable equally to the experiments made with thrombokinase and thrombin. The inhibitory effect of this salt upon fibrin production is therefore manifested in respect of the interaction of thrombin and fibrinogen, the action of thrombokinase upon prothrombin being unaffected. Calcium chloride moreover in the concentrations employed produces from the first retardation of the process of coagulation of fibrinogen by means of thrombin.

If the constants p and n for the experiments with thrombokinase and snake venom are compared as shown below, it will be observed that a striking

	n	Fibrinogen solution employed in each experiment	p 0·325 cc.	p 0·160 cc.	p 0·050 cc.
Thrombokinase (testis of rabbit)	22·12		0·00228	0·0131	—
Thrombin (venom of *Echis carinatus*) ...	34·00		0·026	—	0·0080

difference occurs between the two in respect of the constant p. In the former experiments p increases with diminution of the amount of fibrinogen solution employed; in the latter the reverse occurs. This is readily explicable on the assumption that different kinds of thrombin are present in the two series of experiments. In reality the conditions of experiment are unavoidably complex, for the thrombin present comes from four sources: (1) that produced

by the action of thrombokinase, contained in relatively small amount in the blood plasma of the fowl, upon fowl's prothrombin in presence of calcium chloride; (2) that produced by the action of thrombokinase from the testis of the rabbit upon fowl's prothrombin; (3) that produced by the action of thrombokinase, contained in relatively small amount in snake venom (cf. p. 540), upon fowl's prothrombin; and (4) that contained in snake venom.

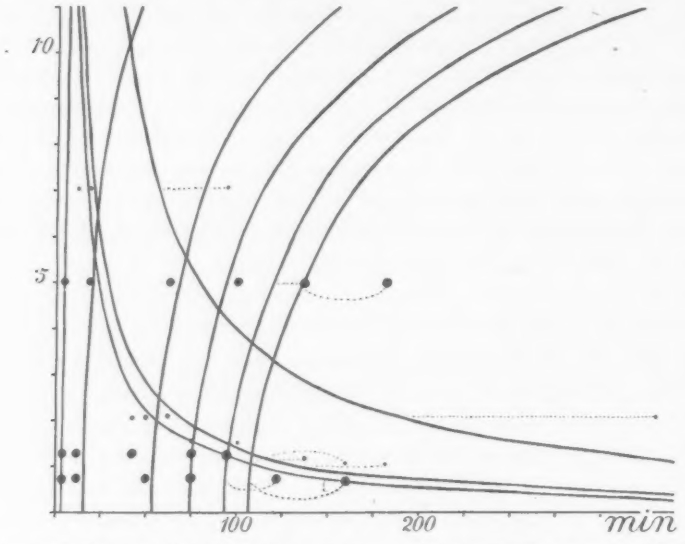

Fig. 6. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables XIII and XV. Abscissae (x), time in minutes; ordinates, cc. of 1 in 300,000 solution of venom (y) for hyperbolic curves (·) or of N/10 $CaCl_2$ (w) for parabolic curves (●). The amount of fibrinogen solution employed in each experiment is 0·325 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0·075 cc. and 0·125 cc. respectively of N/10 $CaCl_2$ solution were employed, the remaining hyperbolic curves to 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 2·94 cc. of venom solution was employed; the remaining parabolic curves to 0·704 cc., 0·209 cc., 0·149 cc., 0·119 cc. and 0·104 cc. respectively.

The chief sources of thrombin are (2) and (4). The enquiry naturally arises, Is the thrombin produced from fowl's prothrombin independent of the source of the thrombokinase employed? The difference in the values of p in the two series of experiments appears to indicate that the thrombin of snake venom is different from that produced by the action of the thrombokinase of the rabbit's testis upon fowl's prothrombin; thrombin produced by the action of the thrombokinase of snake venom upon fowl's prothrombin is

present in too small an amount to enable a safe comparison with the other thrombins under consideration to be made. That the thrombin derived from fowl's prothrombin by the action of fowl's thrombokinase (both contained in the fibrinogen solution employed)—the only auto-thrombin present in these experiments—is not similar to snake venom thrombin is suggested by the circumstance that the more marked divergences between observed and calculated values of x are seen in those experiments in Tables XIII and XIV in which the amount of thrombokinase present is low and the amount of the former variety of thrombin is relatively high, e.g. Exps. 53 and 43 of the second group in Table XIII and similar experiments in adjoining groups.

TABLE XVI.

The experiments recorded in Table XIV rearranged in a form suitable for calculating the values of p and n in the equation $w^2 = p\left(\frac{xy}{z} - n\right)$. *The observed coagulation times are given within brackets in the fourth column.*

No. of experiment	w N/10 $CaCl_2$ solution (cc.)	y Venom solution (*Echis carinatus*), 1 in 300,000 (cc.)	$x_{calculated}$ Time of coagulation (minutes)	xy	$p = \frac{w_1^2 - w^2}{\frac{x_1 y_1}{z} - \frac{xy}{z}}$	$n = \frac{xy}{z} - \frac{w^2}{p}$
2	—	0·453	[4] 3·7	1·70	0·0080	34·00
4	0·075	,,	[4] 3·8	1·73		34·00
6	0·125	,,	[4] 4·0	1·80	,,	33·99
8	0·5	,,	[6] 7·2	3·26	,,	33·95
10	1·5	,,	[31] 34·8	15·75	,,	33·85
14	0·075	,,	[5] 3·8	1·73		34
16	0·125	,,	[5] 4·0	1·80	,,	,,
18	0·5	,,	[8] 7·2	3·26	,,	,,
20	1·5	,,	[40] 34·8	15·75	,,	,,
24	0·075	0·121	[16] 14·3	1·73		,,
26	0·125	,,	[16] 14·9	1·80	,,	,,
28	0·5	,,	[27] 27·0	3·26	,,	,,
30	1·5	,,	[159] 157·4	15·75	,,	,,
34	0·075	0·061	[25] 28·4	1·73		,,
36	0·125	,,	[25] 29·5	1·80	,,	,,
38	0·5	,,	[48] 53·5	3·26	,,	,,
40	1·5	,,	[248] 258·0	15·75	,,	,,
44	0·075	0·031	[49] 35·8	1·73		,,
46	0·125	,,	[55] 58·0	1·80	,,	,,
48	0·5	,,	[93] 105·0	3·26	,,	,,
50	1·5	,,	[510] 507·4	15·75	,,	,,
54	0·075	0·016	[103] 108·1	1·73		,,
56	0·125	,,	[102] 112·4	1·80	,,	,,
58	0·5	,,	[180] 204·0	3·26	,,	,,
60	1·5	,,	[>2500] 985·0	15·75	,,	,,

The same process of reasoning applies when, for the latter variety of thrombin, that produced from fowl's prothrombin by the action of rabbit's thrombokinase (testis) is substituted (Tables IX and X). It is interesting to observe that in the former case the observed coagulation times for low amounts of thrombokinase are longer than the calculated, while in the latter case the reverse occurs.

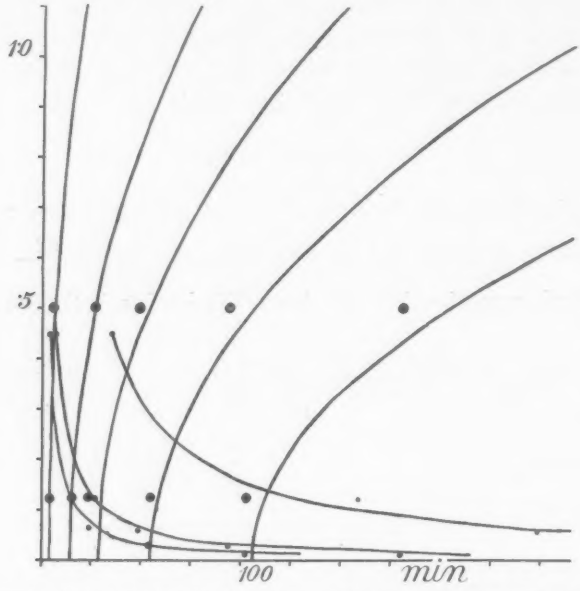

Fig. 7. Hyperbolic (x, y) and parabolic (x, w) curves for the experiments given in Tables XIV and XVI. Abscissae (x), time in minutes; ordinates, cc. of 1 in 300,000 solution of venom (y) for hyperbolic curves (·) or of N/10 $CaCl_2$ (w) for parabolic curves (•). The amount of fibrinogen solution employed in each experiment is 0·050 cc. The hyperbolic curve nearest to the axes represents two curves, corresponding to those experiments in which 0·075 cc. and 0·125 cc. respectively of N/10 $CaCl_2$ were employed, the remaining hyperbolic curves to 0·5 cc. and 1·5 cc. respectively. The parabolic curve nearest to the axis of ordinates corresponds to those experiments in which 0·453 cc. of venom solution was employed; the remaining parabolic curves to 0·121 cc., 0·061 cc., 0·031 cc. and 0·016 cc. respectively.

It has already been pointed out that equation (8)

$$w^2 = p\left(\frac{xy}{z} - n\right)$$

only applies so long as the concentration of calcium chloride does not exceed a limit, which is dependent upon the concentration of fibrinogen. When this limit has been passed, the retardation of coagulation becomes greater than

that calculated from equation (8): in other words, the calculated values of xy are lower than the observed values (cp. the last group of experiments in Tables IX, X, XIII and XIV). In the experiments made with thrombokinase (testis of rabbit) the observed retardation was greater when the concentration of fibrinogen was diminished (0·325 cc. was used in each experiment in Table IX; 0·160 cc. in Table X); with thrombin obtained from venom the observed retardation was more marked when the concentration of fibrinogen was increased (0·325 cc. in Table XIII; 0·050 cc. in Table XIV). This circumstance, which is indicated by the increase of p with diminution of z (fibrinogen) in the former experiments and its decrease with increase of z in the latter, is in its ultimate aspect an index of the physical conditions of experiment. Presumably with an auto-thrombin derived from the same source as the fibrinogen employed, p would increase with decrease of z, while with a hetero-thrombin a smaller increase or an actual decrease would occur.

So far we have only considered those experiments in which calcified venom has been used. If no calcium chloride is employed a retardation of the time of coagulation occurs as is exhibited in the first group of experiments in Tables XIII and XIV; with amounts of calcium chloride considerably below 0·075 cc. in each experiment a less marked retardation occurs. The complete w, x curve for snake venom thrombin is therefore of the type shown in 1, Fig. 8. Any attempt to apply the equation for a hyperbola to this curve is unsuccessful, as is obvious from the aspect of the curve. It appears to consist of two parts: (1) the parabolic curve just described; and (2) a low descending curve. The complete curve thus presents a general resemblance to the former curve furnished by thrombokinase (2, Fig. 8). In both an upper parabolic curve is joined below by a lower flat curve. But here the resemblance ceases, the lower curve obtained with thrombokinase approaching but not touching the axis of abscissae, while that obtained with venom thrombin cuts this axis; the former as already mentioned is due to the action of thrombokinase upon prothrombin in presence of minimal amounts of calcium chloride, the latter is due to an accelerator action of calcium chloride upon venom thrombin, an effect which is produced with very low concentrations of the calcium salt and is speedily followed by the inhibitory

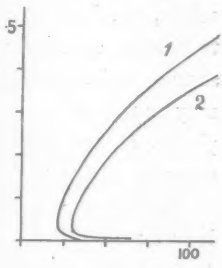

Fig. 8. Type curves. Abscissae, time in minutes; ordinates, calcium chloride. (cc.)

action investigated above. It is important to distinguish this action of calcium chloride in low concentration upon venom thrombin from the action exerted upon thrombokinase in presence of prothrombin contained in the fibrinogen solution employed. In point of fact the venom solution contained thrombokinase, for upon heating to 100° for five minutes a solution was obtained free from thrombin, but containing enough thrombokinase to yield in presence of prothrombin and calcium chloride an amount of thrombin equal to about one-twentieth of that originally present, a quantity which if applied as a correction in Tables XIII and XIV would cause a very slight decrease of some of the calculated coagulation periods. Evidence is thus afforded of the increased coagulative activity of venom thrombin in presence of minimal amounts of calcium chloride. Presumably the same effect under similar conditions of experiment is also produced with thrombin derived from the action of thrombokinase (testis of rabbit) upon fowl's prothrombin.

Before concluding this article further reference may be made to the differences between observed and calculated times of experiment exhibited in the tables. Two sources of these differences, namely (1) that due to the circumstance that thrombin has a maximum effective value and (2) that caused by the concentration of calcium chloride exceeding a limiting value, have already been considered. In addition a certain amount of irregularity is observable, which appears to be at present unavoidable in experiments upon coagulation. Although it is not possible to deal with all the sources of experimental error, some of which appear still to elude detection, while others, e.g. decomposition and evaporation, are variable and especially likely to become effective when the coagulation period is long, nevertheless two sources of irregularity of result to which special attention has been given may be referred to here, namely that due to error of observation of the completion of coagulation and that caused by imperfect measurement of the amounts of the various solutions employed.

The former is a serious difficulty when quantitative work upon coagulation is first undertaken. The problem is to judge when coagulation is complete, that is to say when the whole of the fibrin has separated out. The indications that this has occurred are that the clot has reached (1) its maximum consistence and (2) its maximum opacity; the latter, however, does not appear to be so valuable an indication as the former. Since there is no sharp end point, precise measurement of coagulation time is not possible. Even with the training of observation resulting from the repeated performance of serial experiments, in which the degrees of coagulation in mixtures of slightly

different composition are constantly being compared, the measurements made must be regarded as only approximate. Difficulty is sometimes caused by coagulation being irregular, fibrin separating out to form a framework of strands of varying length and thickness, the fibrils being no longer distributed uniformly throughout the clot; this condition which is occasionally troublesome has been met with chiefly when the amount of fibrinogen has been small, but it sometimes appears when relatively large amounts of fibrinogen have been employed. To judge of the consistence of a clot it is necessary to agitate or shake the tube in which the clot is contained. If this is not done gently a soft clot may be detached before complete separation of fibrin has occurred; contraction of the clot may then take place followed by the appearance of a second coagulum, usually of soft consistence, in which case the determination of the coagulation time cannot be made with much approach to precision.

A second source of error arises from the difficulty of delivering accurately the small amounts of fluid which must necessarily be dealt with when large numbers of experiments have to be made with limited quantities of fibrinogen solution. It must be borne in mind that it is not the amount of fluid which is *contained* in the measuring pipette, but the quantity which is actually *delivered*, which requires accurate measurement. Consequently the amounts actually delivered must be determined and also the extent of the variations occurring between successive deliveries from the same scale mark. When this has been done the degree in which the time of coagulation is likely to be affected by unavoidable error of delivery can be estimated with the aid of the data given in the tables. The tendency of fluid to adhere to glass in the neighbourhood of the mouth of a pipette may be overcome by coating the outside of the end of the pipette with hard paraffin. Any greasy material present on the inner surface of the pipette interfering with filling and delivery is best removed by heating to a temperature just sufficient to cause any organic matter present to become charred; the inner surface of the pipette is then readily wetted by water. In performing a series of experiments each pipette should be used exclusively for one constituent of the mixture of liquids employed; if used for several constituents in succession, very careful cleaning may not suffice to avoid contamination. In practice, when performing serial experiments similar to those recorded in the tables, it will be found convenient to prepare a series of dilutions of calcium chloride such that an equal amount of fluid is delivered in each experiment, the same pipette, filled to the same scale mark, being used for each tube, proceeding from lower

to higher concentrations, and being washed out several times with each liquid of higher concentration before commencing to deliver. The solutions of thrombokinase were similarly prepared and dealt with. In order to destroy traces of thrombokinase remaining adherent to the inner surface of the tubes after coagulation has been completed, the latter were, after being previously cleaned, heated just below redness before being again employed.

In conclusion I desire to express my indebtedness to Dr C. J. Martin for the valuable assistance he has given me in connection with this research.

Summary.

(1) A given amount of thrombokinase, in presence of calcium chloride and of a sufficient amount of prothrombin, will produce a definite quantity of thrombin, which is independent of the actual concentration of prothrombin.

(2) In experiments with thrombokinase the coagulation time is concerned with the action of thrombin upon fibrinogen.

(3) The action of thrombin (y) upon fibrinogen (z) when a decinormal calcium chloride solution is present to the extent of 0·05 cc. in a total volume of 0·5 cc. proceeds approximately according to the equation

$$xy = nz,$$

where x = coagulation time and n is a constant.

(4) The effect of adding decinormal calcium chloride solution, in amounts exceeding 0·05 cc. per 0·5 cc. of fluid, to (1) snake venom thrombin or (2) a mixture of thrombokinase and prothrombin is to cause retardation of coagulation of fibrinogen. If the coagulation times (x) are plotted against the amounts of calcium chloride employed, the points so obtained are found to lie on a parabola given by the equation

$$w^2 = ax - b,$$

so long as the amount of calcium chloride lies below a critical limiting value, beyond which this equation is no longer valid. Of the two constants in the equation a is proportional to the amount of thrombokinase present; b is a function of z, whose form has not been determined.

(5) The above action of thrombin upon fibrinogen takes place according to the general equation

$$w^2 = p\left(\frac{xy}{z} - n\right),$$

where n is a constant, independent of w, xy or z, and p is a function of z, not yet determined.

(6) Minimal quantities of calcium chloride (i.e. amounts not exceeding 0·05 cc. of a decinormal solution, contained in a total volume of fluid amounting to 0·5 cc.) accelerate the coagulant activity of thrombin furnished by the venom of *Echis carinatus*.

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L. ON THE EFFECTS OF VARIOUS SUBSTANCES (ELECTROLYTES, NON-ELECTROLYTES, ALKALOIDS, ETC.) UPON THE UREASE OF SOY-BEAN.

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(*Received November 15th, 1915.*)

Musculus [1874] was the first to discover the ferment urease, which is capable of decomposing urea into NH_3 and CO_2, in urine. Shibata found urease in *Aspergillus niger* [1904], and Kikkoji in *Cortinellus edodes* [1907]. Takeuchi was the first to discover urease in soy-bean in considerable amount [1909]. Various workers in succession [Keisel, 1911; Zemplén, 1912; and Annet, 1914] succeeded in finding urease in varying amounts in the seeds of various higher plants (wheat, lupin, some Indian seeds, etc.). Subsequently Marshall [1913, 1 and 2] and Plimmer and Skelton [1914] used the urease of soy-bean to estimate urea in urine, blood, etc., with extreme accuracy. These investigations were followed by the study of the mode of action of urease and the conditions which facilitate or retard its action.

Takeuchi endeavoured to ascertain the extent of the influence of salts, acids and caustic soda on urease. But we find his data of the concentration of the substances used by him too high, owing to the lack of an accurate method of estimation.

Armstrong and Horton [1912] and Armstrong, Benjamin and Horton [1913] studied the influence of various substances upon urease. They found that urea at a concentration of more than M/5 depressed the development of NH_3, and all acids, even such relatively weak acids as carboxylic acids, acted on urease in an inhibitory manner, with the exception of amino acids and carbon dioxide which accelerated the action of urease. Boric acid was

inhibitory at M/50 and phenol at M/5. These observers also found that alcohols, aldehyde and glucose act as inhibitors; as regards alcohols, the lower their solubility, the greater was their inhibitory effect.

Marshall [1914] found that the velocity of action of urease is dependent on the hydrogen or hydroxyl ion concentration within rather narrow limits. Hydrochloric acid and caustic soda in sufficient amounts inhibited or destroyed urease. Ethyl alcohol had a moderate inhibitory effect but had apparently a slight destructive action upon urease. It markedly retarded urease action in concentrations over 20 volumes per cent.

Armstrong and his collaborators, and Marshall, used the water extract of soy-bean powder as urease. This extract contains a certain amount of buffer substances, such as albumin, which disturb the accurate neutralisation of the extract by means of titration. This was recognised more or less by these authors, and when they desired to ascertain the effect of dilute acids upon urease, these acids were mixed with urea and urease in the digestion bottle. It was found that the acids added in small quantities may be neutralised after a short time by the development of NH_3.

To avoid the difficulty due to certain impurities, van Slyke and Cullen [1914, 2] tried successfully to obtain urease in a purer state by precipitating the water extract of soy-bean powder with acetone and drying the precipitate in vacuo over sulphuric acid. This dried precipitate, dissolved in distilled water before use, was used as urease. Van Slyke and his co-workers [van Slyke and Cullen, 1914, 1; van Slyke and Zacharias, 1914] also studied the effect of hydrogen ion concentration upon urease, altering the former as required by means of a phosphate mixture. They regarded the urease reaction as proceeding in two stages characterised by different velocities, the first being the combining velocity (c) of the urease and substrate, and the second the decomposing velocity (d) of the combined substance into NH_3 and CO_2. The more alkaline the reaction of the medium, the greater was c, but d was maximal at the neutral reaction and less when the reaction was more alkaline or more acid. The total action of urease was depressed at the slight acid reaction of $P_H = 6{\cdot}07$ and reached its maximum when P_H was close to 7. The authors ascribed the depressing effect of the product of the action to its hydrogen ion concentration, because the further addition of ammonia to the medium is no longer able to depress the urease action, since the hydrogen ion concentration reaches a certain limit and remains constant in spite of the increase of ammonia. Neutral salts, glucose and ethyl alcohol retarded c and at a high osmotic pressure retarded d as well.

Armstrong and Horton [1912] found that the velocity of action of urease proceeds independently of the amount of the substrate remaining unchanged, provided that the product with retarding effects on urease is neutralised.

Van Slyke and his collaborators introduced a formula for general use in enzyme action, making use of c and d. This formula is as follows:

$$t = \frac{1}{E}\left(\frac{1}{c}\log\frac{a}{a-x} + \frac{x}{d}\right).$$

They recommended this formula as the most accurate one because it corresponded exactly to the experimental data. They alleged that urease was not a contact catalyst but formed an intermediate labile compound with urea. They insisted that the action of urease was in accordance with the law of mass-action.

My experiments were directed towards ascertaining whether the hydrogen ion concentration alone is adequate to account for the effect of acids upon urease, as the custom is growing of stating results on enzyme action in terms of hydrogen ion concentration. This tendency has been increased by the work of Michaelis and his co-workers, and of Sörensen, etc. If enzymes are colloids and the reactions take place at the surface of colloids, it is reasonable to consider other physico-chemical properties at the same time as osmotic pressure and hydrogen ion concentration. Among the physico-chemical properties surface tension is the first to come into consideration. According to the Gibbs-Thomson theorem, substances which lower the surface tension of solutions accumulate at the surface of the second phase, e.g. at the boundary between air and solution or at the interface between suspended particles of solid and the solution, or between droplets of an immiscible fluid and the solution. Thus substances which actively affect surface tension may influence in one or other direction the actions which have some relation to surface. They may have an effect on enzyme particles in the sense of diminution or increase of active surface, i.e. aggregation or dispersion. They may also have effects on diffusion, absorption, adsorption, internal friction, etc. (see Traube's reports). To solve these questions various acids, alcohols, etc., which alter the surface tension of the solvent, were examined. I also endeavoured to ascertain whether the depressing effect of ammonium carbonate on urease should be ascribed to the hydroxyl ion concentration alone or in part to the reversible reaction of the hydrolysis of urea.

Experimental Part.

Methods.

Urease solution was mixed with urea in digestion bottles. After some hours, acid of considerable concentration was added in order to stop the urease action instantaneously. The ammonia developed on subsequent addition of sodium carbonate was driven off by means of an air current free from ammonia and caught in decinormal sulphuric acid. The absorbed ammonia was estimated by the usual method of titration by N/10 caustic soda.

The experiments were always carried out at room temperature. The digestion bottles were placed in a large wooden water bath in a position in the laboratory where the change of air temperature was as small as possible. During the period of several months in which the experiments were carried out, the temperature of the bath varied between 19° and 21°. The change of temperature during each experiment was very slight.

As digestion bottles I used the high cylinders used by Plimmer in his work on urea estimation by means of urease. The inner diameter of these cylinders is about 4·2 cm., and their height about 40 cm. Cylinders were selected which had the same thickness of wall approximately.

The Allihn bottles, into which the developed ammonia was led to combine with decinormal sulphuric acid, are about 17 cm. high, with a content of 400 cc. The tubes which lead the ammonia to the sulphuric acid have six small holes at the closed end, each hole having a diameter of about 1·5 mm. Air with ammonia is bubbled through these holes into the sulphuric acid. The faster the air containing ammonia is drawn through these holes, the smaller are the bubbles and the more accurate is the ammonia estimation, as Folin, van Slyke and Plimmer ascertained.

In my experiments the urea was used in a concentration of 0·15 to 0·12 % of the whole mixture in the digestion bottle. This concentration of urea is the most favourable for urease action (van Slyke).

In order to check the urease action instantaneously sufficient N/5 sulphuric acid was added to the bottles to make the whole at least decinormal after neutralisation of the ammonia present. This checking action was complete in half an hour, but the bottles were always left for one or two hours. The mixture was then neutralised by the addition of 2N caustic soda and crystalline sodium carbonate added to make a 4 % solution. Folin's aeration was carried out in the water bath at a temperature of 40°.

The N/10 sulphuric acid which was to catch the ammonia was made up with distilled water to 250 cc. and alizarin red was used as indicator. To prevent frothing a small quantity of lubricating oil was poured into the bottles. The escape of ammonia was prevented during the urease action by the use of rubber corks or ordinary corks and wax. Unless specially mentioned the total quantity of acting medium was always 50 cc. Each digestion bottle contained 5 cc. of 1·5 % solution of urea, with the exception of one series of experiments to be mentioned later in the experimental part. If the action of urease is complete with this amount of urea, then theoretically 25 cc. of N/10 ammonia should be evolved.

Two different preparations of urease were used, one being the 1–10 % water extract of soy-bean powder. This powder was digested carefully in distilled water, left half an hour, with occasional stirring, and filtered through linen. Only the fresh filtrate was used. The other solution was that of precipitated urease prepared by van Slyke's method. This solution was always made immediately before use and the undissolved flocculent substance was filtered off.

The reaction of the water extract of soy-bean was faintly acid compared with that of the standard neutral solution of Prideaux [1912], alizarin red being used as indicator. This extract did not turn phenolphthalein red, as Armstrong stated. Marshall, however, found the water extract of soy-bean alkaline to methyl orange.

0·1 cc. of N/10 ammonia was evolved from 10 cc. of 10 % water extract of soy-bean after standing for 20 hours at room temperature. This correction is made in the experimental data.

The reaction of the solution of the precipitated urease was found by van Slyke to be acid, but in my preparation it was quite neutral.

In order to make certain of my technique of ammonia estimation I carried out the following preparatory experiments.

I made up a N/5 NH_4Cl solution and by Kjeldahl's method I found 19·6 cc. N/10 ammonia in 10 cc. of this NH_4Cl solution. Folin's method was carried out in the water bath at a temperature of 15° for three hours, and the amount of N/10 NH_3 obtained was only 16·98 and 18·20 cc. instead of 19·6 cc.

In the next experiment Folin's distillation of ammonia was carried out in the water bath at a temperature of 40° for an hour, an hour and a half, and two hours respectively. The amounts of N/10 NH_3 obtained were 19·65, 19·69 and 19·66 cc. respectively. We know thus that all the ammonia was

driven out under these conditions within an hour. In all the following experiments the distillation lasted for an hour and a half to two hours.

To check the urease action instantly, I always used sulphuric acid in my work, and avoided the use of heat. It is known that boiling of urea solution with acid or alkali develops ammonia to a small extent [see Armstrong and Horton, 1912].

To 5 cc. of 1 % bean extract N/5 H_2SO_4 was added to make N/10 and N/50 solution *in toto*. The acid was allowed to act on urease fifteen minutes, then the mixture was neutralised with N/5 NaOH. Urea was then added, the mixture was kept at room temperature the whole night, and NH_3 was determined in this mixture exactly as described previously. The control mixture without acid developed 23·83 cc. of N/10 NH_3. The urease inhibited by N/10 H_2SO_4 developed only 0, 0·03, 0·11, 0·05 cc., an average of 0·05 cc. of N/10 NH_3, and urease inhibited by N/50 H_2SO_4 developed only 0·06, – 0·02 cc., an average of 0·02 cc. of N/10 NH_3. Notwithstanding this fact, in order to check urease action, I always added to the digestion bottles N/5 H_2SO_4 so as to make the whole mixtures stronger than N/10 and left them for half an hour to one hour.

Urease mixed with 4 % crystalline sodium carbonate developed no ammonia at 40° in several hours.

(1) *Effects of acids on urease.*

Marshall found that the velocity of urease action is dependent upon the hydrogen or hydroxyl ion concentration within rather a narrow limit. Van Slyke found that slight acidity ($P_H = 6·07$) exercised a depressing effect on urease action and that the velocity reached its maximum at the neutral point ($P_H = 7$).

I made my first experiments with a water extract of soy-bean as did Armstrong and Marshall. Before use the bean extract had been neutralised with N/20 NaOH, being compared with the standard solution of Prideaux.

The acids employed were prepared by titration against N/10 NaOH, using phenolphthalein as an indicator. Only phosphoric acid was prepared by weighing from Merck's pure preparation, and this was estimated by weight after being converted into magnesium pyrophosphate.

The first experiment was commenced with sulphuric acid; the acid was mixed in digestion bottles with 5 cc. of 1·5 % urea solution and 10 cc. of a 2·5 % extract of the bean and left for nearly 20 hours at room temperature. Urease action was then checked and NH_3 estimated.

We see from this experiment that sulphuric acid is capable at a concentration of N/100 and N/500 of totally inhibiting urease, while at a concentration of N/1000 and N/1500 it has practically no effect.

In order to ascertain to what extent the enzyme was inactivated by the prolonged action of acid, the urease and acid were mixed, left for some time and then neutralised with NaOH before the addition of urea.

TABLE I.

H_2O in cc.	H_2SO_4 in cc.	Conc. of acid	N/10 NH_3 developed in 17 hrs in cc.	
35	—	—	24·21	24·14
35	—	—	24·06	
30	N/10 5	N/100	0·02	0·12
30	N/10 5	N/100	0·11	
30	N/50 5	N/500	0·29	0·25
			0·20	
10	N/500 25	N/1000	24·08	
10	N/750 25	N/1500	23·98	

Sulphuric acid was allowed to act on urease for 16·5 hours, then neutralised, mixed with urea and left for 22·5 hours at room temperature. The urease action was then checked and the NH_3 estimated. The control urease developed 24·42 cc. of N/10 NH_3. N/500 sulphuric acid was found to have totally inactivated the urease; and N/1000 sulphuric acid had almost completely inactivated the urease, only 0·5 cc. of N/10 NH_3 being formed.

In order to find out the lower limit of the inactivating effect of sulphuric acid the experiment recorded in Table II was carried out. The columns of this table read from left to right show the order in which the various substances were added to the digestion bottles. In every experiment the procedure was carried out as Tables I and II.

TABLE II.

H_2O in cc.	1 % bean extract in cc.	N/200 H_2SO_4 in cc.	Conc. of acid	Added after 17 hours: H_2O in cc.	Added after 17 hours: N/200 NaOH in cc.	Added after 17 hours: 1·5 % urea in cc.	N/10 NH_3 developed in 20·5 hrs. in cc.	
40	10	—	—	10	—	5	24·05	24·16
40	,,	—	—	,,	—	,,	24·27	
30	,,	10	N/1000	—	10	,,		0·10
33	,,	7	N/1429	3	7	,,		0·62
35	,,	5	N/2000	5	5	,,		13·86
36	,,	4	N/2500	6	4	,,		23·98
37	,,	3	N/3300	7	3	,,	24·12	24·03
37	,,	,,	,,	,,	,,	,,	23·93	
38	,,	2	N/5000	8	2	,,	24·15	24·04
38	,,	,,	,,	,,	,,	,,	23·93	

When the added sulphuric acid was not neutralised by the NH_3 developed, a markedly depressing effect of N/2000 H_2SO_4 in 17 hours was observed, N/2500 acid showed some depressing effect, whilst N/3330 had no effect.

It is to be noticed here that acids, whether organic or inorganic, caused in urease solution an opacity or fine flocculent precipitation. Even N/5000 H_2SO_4 caused a marked opacity, which disappeared on neutralisation. If this neutralised mixture of acid and urease still contains some effective urease, and consequently ammonia is developed, the mixture goes into the turbid state again.

By the same method as that mentioned above, the influence of hydrochloric acid, acetic acid and butyric acid on urease was examined.

TABLE III.

	N/10 NH_3 developed in 20·5 hrs in cc.		
Concentration of acid	HCl	Acetic acid	Butyric acid
N/1000	—	15·87	18·99
N/2000	16·09	24·06	23·81
N/2500	24·17	24·29	23·50
N/3330	24·12	24·28	23·78
N/5000	—	24·38	23·82
Control urease without acid	24·25	24·38	23·76

By these experiments it was proved that all acids employed at a strength of N/2500 did not affect urease.

If we want to compare the effect of more diluted acids on urease it is necessary to use precipitated urease instead of water extract of soy-bean, because the latter contains much regulatory substance, which may protect urease from being inhibited by diluted acid.

All the following experiments, unless there is some note otherwise, were carried out with a solution of precipitated urease. At first I endeavoured to obtain some idea of the relative strength of various acids against urease.

N/500 acids were allowed to act on 5 cc. of 0·5 % urease for 17 hours, when they were neutralised, mixed with urea and left at room temperature for 22·5 hours. The amount of NH_3 evolved from these mixtures is shown in Table IV.

The NH_3 developed was very small in amount, proving that even weak acids are able to destroy urease in process of time.

In order to find out the length of time necessary for the action of acids on urease, N/500 acids were allowed to act on 5 cc. of 0·5 % urease for 15,

TABLE IV.

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone	23·62	23·60
" "	23·59	
HCl	0·18	0·19
"	0·19	
Oxalic	0·22	0·22
"	0·21	
Formic	0·22	0·21
"	0·20	
H_2SO_4	0·33	0·28
"	0·22	
H_3PO_4	0·33	0·27
"	0·21	
Acetic	0·36	0·36
"	0·35	
Butyric	0·41	0·37
"	0·32	

30, 60 and 90 minutes, the acids were then neutralised, urea was added and the mixture left at room temperature for 16·5 hours. Urease action was then checked and NH_3 estimated.

TABLE V.

Acids neutralised after	Developed N/10 NH_3 in cc.	
	H_2SO_4	CH_3COOH
15 minutes	0·87	—
30 "	0·61	5·64
60 "	0·26	4·50
90 "	0·20	1·65
0·5 % urease alone	23·81	

We see here a very distinct difference between a strong and a weak acid in regard to the velocity of their inhibitory effect. Regarding these facts, I varied the length of time of exposure of urease to acids and the strength of acids employed. A number of experiments were carried out, the following acids being used: HCl, HNO_3, H_2SO_4, H_3PO_4, formic, acetic, butyric, oxalic. trichloracetic, benzoic and salicylic.

TABLE VI.

N/1000 *acids were allowed to act upon* 5 *cc. of* 0·5 % *urease for* 10 *minutes, then neutralised, mixed with urea and left at room temperature for* 16 *hours.*

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone	23·85	23·82
" "	23·79	
Trichloracetic	0·48	0·54
"	0·60	
Oxalic	0·38	0·56
"	0·73	
HCl	0·61	0·61
"	0·61	
Formic	2·40	2·29
"	2·17	
Butyric	23·69	23·47
"	23·24	
H_3PO_4	23·56	23·63
"	23·69	
Acetic	23·80	23·76
"	23·72	

TABLE VII.

N/1000 *acids were allowed to act upon* 5 *cc. of* 0·5 % *urease for one hour, then neutralised, mixed with urea and left at room temperature.*

Name of acid	Developed N/10 NH_3 in 22 hrs in cc.
H_3PO_4	3·06
Butyric	11·40
Acetic	15·02
	Developed N/10 NH_3 in 21 hrs in cc.
Salicylic	0·66
Benzoic	1·14
Acetic	15·19

TABLE VIII.

N/2000 *acids were allowed to act upon* 5 *cc. of* 0·5 % *urease for one hour, then neutralised, mixed with urea and left at room temperature for* 20·5 *hours.*

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone		23·55
HNO_3	0·49	0·42
"	0·34	
Trichloracetic	0·48	0·43
"	0·38	
HCl	0·50	0·50
"	0·49	
H_2SO_4	0·65	0·83
"	1·01	
Salicylic		1·27
Formic		3·90
Benzoic		19·02

TABLE IX.

N/3000 *acids were allowed to act upon* 5 *cc. of* 0·5 % *urease for an hour, then neutralised, mixed with urea and left at room temperature for* 18 *hours.*

Name of acid	Developed N/10 NH_3 in cc.	
0·5 % urease alone		23·87
Oxalic	21·47	20·83
,,	20·18	
Trichloracetic		21·15
HCl	23·15	23·26
,,	23·36	
HNO_3	23·37	23·50
,,	23·62	
H_2SO_4	23·58	23·62
,,	23·66	
Formic	23·92	23·76
,,	23·60	

TABLE X.

N/4000 *acids were allowed to act upon* 5 *cc. of* 0·5 % *urease for an hour, then neutralised, mixed with urea and left at room temperature for* 20·5 *hours.*

Name of acid	Developed N/10 NH_3 in cc.
0·5 % urease alone	24·35
Oxalic	21·22
H_2SO_4	23·57
HNO_3	23·63
HCl	23·72
Trichloracetic	23·77
Formic	23·78

If we arrange the acids according to their inhibitory effect upon urease, we get the following order.

The inhibitory effect decreases passing from left to right.

TABLE XI.

According to Table VI:
Trichloracetic, oxalic, hydrochloric > formic > butyric, phosphoric, acetic.
According to Table VII:
Salicylic > benzoic > phosphoric > butyric > acetic.
According to Table VIII:
Nitric, trichloracetic, hydrochloric > sulphuric > salicylic > formic > benzoic.
According to Table IX:
Oxalic > trichloracetic > hydrochloric, nitric, sulphuric, formic.
According to Table X:
Oxalic > sulphuric, nitric, hydrochloric, trichloracetic, formic.

If we arrange the above-mentioned acids according to the strength of their inhibitory effects on urease, we get a general order of acids from Table XI as follows:

Oxalic > Trichloracetic > Hydrochloric, Nitric > Sulphuric > Salicylic > Formic > Benzoic > Phosphoric > Butyric > Acetic.

According to Ostwald, if one gram-molecule of each of these acids is dissolved in 1024 litres of distilled water, they are dissociated at 25°, as follows:

Trichloracetic acid	...	...	99·40 %	
Salicylic acid	...	...	62·80 %	
Formic acid	...	...	35·80 %	
Benzoic acid	...	...	21·61 %	
Acetic acid	...	...	12·61 %	
Butyric acid	...	...	11·41 %	
Oxalic acid	...	...	94·70 %	(gram-molecule in 256 litres).

This order of hydrogen ion concentration of nearly N/1000 acids corresponds with the order of acids which I obtained in Tables VI, VII and VIII. But there is one important exception, namely, butyric acid (Tables VI and VII). This, in spite of a lower hydrogen ion concentration than acetic acid, had an inhibitory effect on urease greater than that of acetic acid. This fact corresponds precisely with the result of J. Loeb [1909], who found butyric acid to have a stronger influence on membrane construction of sea-urchin eggs even than mineral acids. He remarked that acids in their physiological action are not dependent only upon their hydrogen ion concentration, and he is of opinion that the undissociated part of an acid plays the main rôle concerning its relative permeability through the cell membrane of eggs, and that the process may be of a purely physical nature.

According to Ostwald the equivalent conductivity of N/1000 acids at 18° is as follows:

HCl	375 practically totally dissociated,
HNO_3	373,
$1/2H_2SO_4$	355,
$1/3H_3PO_4$	106,
CH_3COOH	41.

If we compare the order of acids obtained in this work with the degree of dissociation of acids given above, we see that the former coincides with the latter fairly well.

At a dilution of N/3000 and N/4000 it is considered that the strong mineral acids such as HCl, HNO_3 and H_2SO_4 are almost entirely dissociated. Organic

acids with high dissociation coefficients, as trichloracetic acid and oxalic acid, will be in the same dilution also almost entirely dissociated. Yet I have confirmed, by Prideaux's colorimetric method, that they have a smaller hydrogen ion concentration than HCl in the same dilution. Notwithstanding, we find a markedly stronger inhibitory effect of trichloracetic and oxalic acid than mineral acids. These exceptional cases force us to take other considerations than the exclusive idea of hydrogen ion concentration into account.

It is true that the hydrogen ion concentration of acids plays the main rôle in many purely chemical reactions. According to Ostwald, the inversion order of acids is: HCl, HNO_3, H_2SO_4, tri-, di-, mono-chloracetic acid, formic acid, acetic acid. It is just the order of hydrogen ion concentration. On the other hand there are many contradictions with regard to the physiological actions of acids.

Paul, Birnstein and Reuss obtained the order of acids as follows, classified by their bactericidal action [see Traube, 1912]:

Trichloracetic acid, HNO_3, HCl, oxalic acid, H_2SO_4, H_3PO_4, formic acid, acetic acid, butyric acid.

Barratt [1904] found that univalent aliphatic acids are more toxic than mineral acids on *Paramoecium*. Führer and Neubauer [1907] showed that the limiting concentration of acids capable of causing haemolysis does not correspond to their hydrogen ion concentration.

Pauli [1910] investigated the order of acids in regard to their influence on the internal friction and the precipitability of solutions of albumin by alcohol, and found it to be:

Trichloracetic acid, dichloracetic acid, H_2SO_4, HNO_3, HCl, monochloracetic acid, acetic acid.

He ascribed these effects not to the strength of acids, but to the nature of their anions.

Loeb's experiments on parthenogenesis have been mentioned already. Here again the order of hydrogen ion concentration is not the order of activity. As to the results of the present work it shows us that the effect of acids on urease is mainly dependent upon hydrogen ion concentration, but not upon negative ions, because the influence of salts of these acids is not apparently so strong, as my later experiments and van Slyke's "salt effect" show. But on the other hand my experiments show us that there are some exceptions to the order of hydrogen ion concentration, viz. butyric acid, trichloracetic acid and oxalic acid.

By these facts we are forced to consider as important the properties of acid radical ions, or undissociated acids, which may act in the way of facilitating the contact of enzyme particles and hydrogen ions.

Among the physical properties of acids or acid radical ions the surface tension is perhaps the most important, assuming that enzyme action takes place at the surface of enzyme particles.

According to Traube's [1909] measurements the constant of surface tension (γ) of molecular solutions of acids is as follows. The constant $\frac{rhs}{2}$ is given in mg./mm. and was measured at 15°. The constant (γ) for water was taken as 7·30:

acids:	$\frac{1}{2}H_2SO_4$	HCl	HNO_3	formic	acetic	propionic	butyric	trichloracetic
γ:	7·285	7·23	7·19	6·84	6·05	4·82	3·31	4·82

It is known that the less the surface tension the greater is the partition coefficient and adsorption coefficient. In adsorption, the adsorbed substances can carry other molecules or ions with them. According to these considerations the inhibitory action of acids on urease is, I assume, a function of hydrogen ion concentration and of surface tension, which latter gives the ions more opportunity of combining with urease particles in the diluted condition. The greater the lowering of surface tension, the more will the relatively smaller hydrogen ion concentration of the weaker acids be compensated by having more opportunity to come into touch with the urease particles.

By means of these considerations the exceptional cases of butyric and trichloracetic acids are explained. Many results concerning the biological effects of acids on bacteria, haemolysis, paramoecium, colloidal precipitation and parthenogenesis will be found to receive a common explanation in this way.

There is still only one difficulty to explain, that is, the superior efficiency of oxalic acid. This acid is not one with a very strong surface-tension activity as is trichloracetic acid. Some other factors, probably physical ones, remain to be detected to explain this exception.

(2) *Effects of caustic soda and ammonia on urease.*

Takeuchi, Armstrong and Marshall reported the inhibitory effects of alkalis on urease. Van Slyke and Zacharias [1914, p. 208] allege that the hydroxyl ion retards *d* but facilitates *c*. In accordance with this the optimum of urease action in diluted urea solution is more on the alkaline side, because in the diluted urea solution the combination of urease and of urea is retarded.

At first I experimented with neutralised 2 % watery extract of soy-bean. To 5 cc. of this extract caustic soda and urea were added, bringing the content of each bottle to equal volumes, the mixture left at room temperature for twenty hours, the urease action then stopped and ammonia estimated.

TABLE XII.

Concentration of NaOH	Developed N/10 NH_3 in cc.
2 % bean extract alone	19·93
N/10	0·18
N/50	0·26
N/100	1·47
N/500	0·95

We see here that caustic soda inhibited urease action even at a concentration of N/500.

By the same method an experiment was made with ammonia. From the quantity of ammonia obtained the known quantity of ammonia added was subtracted with the following result.

TABLE XIII.

Concentration of NH_3	Developed N/10 NH_3 in cc.
2 % bean extract alone	24·32
N/20	12·35
N/50	21·46
N/100	24·05
N/200	24·50

We see here that the inhibitory action of ammonia is much inferior to that of soda. N/50 ammonia had a slight depressing effect.

I have also examined the effect of soda on precipitated urease.

TABLE XIV.

NaOH was allowed to act upon 5 cc. of 0·5 % urease for 1·5 hours, then neutralised, mixed with urea and left at room temperature for 22 hours.

Concentration of NaOH	Developed N/10 NH_3 in cc.
0·5 % urease alone	22·85
N/50	0·16
N/100	8·60
N/250	18·67
N/500	21·00
N/1000	22·77

It was thus found that N/50 soda totally destroyed the urease in 1·5 hours, while N/500 soda had a slight action and N/1000 soda had no effect.

If we compare the results obtained in Tables XIII and XIV we see that the inhibitory effect of N/500 soda and N/50 NH_3 are about equal. But soda was allowed to act on urease only 1·5 hours, while N/50 NH_3 was left for 20 hours with urease. Thus we can assume with certainty that the effect of N/500 soda must be greater than that of N/50 NH_3.

Marshall, and van Slyke and his co-workers, ascribed the depressing effect of alkalis and ammonia on urease to their hydroxyl ion concentration. But there is another possibility that the hydrolysis of urea by urease may be a reversible one.

According to Kohlrausch, the equivalent conductivity at 18° is nearly 204 for N/500 NaOH, only 3·3 for N/10 NH_3 and 9·6 for N/100 NH_3. It is evident that we cannot explain the effect of alkali and ammonia on urease only by hydroxyl ion concentration. The difference of surface tension of soda and ammonia is also not enough to afford the required explanation.

We may therefore suggest that the effect of soda may be the effect of hydroxyl ion concentration and the effect of ammonia be partly due to the reversible reaction of hydrolysis of urea by urease, but this latter possibility is very doubtful.

(3) *Effects of neutral salts and alkaloids on urease.*

As is seen in the previous experiments the depressing effect of ammonia was far below that of soda. We may expect therefore that if neutral salts of strong alkalis be added to the digestion bottle and their bases be displaced by ammonia, the more or less depressing effect on urease, according to the kind of alkali, can be seen.

Van Slyke and his coadjutors found that neutral salts retard *c* and, if their concentration exceeds a 2 M solution, also retard *d*. If my assumption is right, van Slyke should have obtained the result that neutral salts retard *d* and facilitate *c*, because in my opinion alkalis displaced by ammonia will have an influence on urease.

In order to investigate this matter the following experiments were made.

At first neutral salts were mixed with 5 cc. of 0·5 % urease and urea, and after standing at room temperature for 20 hours urease action was checked and ammonia was estimated. It was noticed that the mixture showed a fine flocculent precipitation directly after adding $CaCl_2$, $BaCl_2$, and $HgCl_2$ to urease.

TABLE XV.

N/10 salt solution	Developed N/10 NH_3 in cc.	
0·5 % urease alone	22·73	22·78
" "	22·82	
NaCl	20·32	19·85
"	19·38	
$CaCl_2$	22·03	22·18
"	22·32	
$BaCl_2$	22·68	22·20
"	21·72	

As I supposed N/10 NaCl has an apparent depressing effect while N/10 $CaCl_2$ and N/10 $BaCl_2$, in spite of precipitation, showed only a slight depressing effect. We can understand the marked depressing effect of 2 M solutions of neutral salts on urease as observed by van Slyke.

In the salt solutions of the present experiment the number of chlorine ions are equal, therefore these different depressing effects of neutral salts must be ascribed to the nature of the metallic ions. If the action of metallic ions is a tendency to colloidal precipitation of urease, we should expect more effect of the multivalent ions as $Ba^{\cdot\cdot}$ and $Ca^{\cdot\cdot}$ than of the univalent $Na^{\cdot}$ ions. This was not the case.

It was concluded therefore that the metallic bases of these neutral salts were displaced by ammonia hydrolysed from urea, owing to the law of mass-action. Metallic bases are able to dissociate more hydroxyl ion than their equivalent of ammonia. As to the degree of dissociation, sodium stands of course far beyond barium and calcium.

Van Slyke reported that neutral salts disturb the combination of enzyme and substrate, so that, when the concentration of urea becomes smaller at the end of the reaction, the depressing effect of neutral salts on the combination of urea and enzyme becomes more manifest.

If the urease action is checked at an early stage, then one would see less effect of neutral salts. The data of Table XVI agree with this assumption.

TABLE XVI.

N/10 salt solution	Developed N/10 NH_3 in 17·5 hours in cc.
0·4 % urease alone	12·10
NaCl	5·73
$BaCl_2$	5·52
$CaCl_2$	5·62
$HgCl_2$	0·00

But we can explain this result otherwise, assuming that hydroxyl ion concentration is effective on urease only when it exceeds some limit.

In an early period of the reaction the hydroxyl ion concentration would not perhaps reach the effective limit. That is the reason why we do not see in Table XVI a distinct difference between Na, Ba and Ca ions in their effects.

$HgCl_2$ totally destroyed urease, as Hata [1909] found in the case of various ferments; it is to be ascribed to the special nature of the mercuric ion. I did not try to restore the lost urease action by K_2S.

Necessarily we are forced to ask, What will be the result if we use salts of weaker alkaline substances than ammonia? We can expect with reason to see accelerating effects. I used for this purpose the salt of an alkaloid (quinine hydrochloride).

TABLE XVII.

H_2O in cc.	1 % quinine hydrochloride in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 17 hrs in cc.
40	—	5	5	10·65
35	5	,,	,,	13·25
37·5	2·5	,,	,,	11·74
39	1	,,	,,	11·49

After standing for 17 hours the mixture in the digestion bottles became turbid. It indicates that quinine was precipitated, being freed from its salt by the action of the ammonia developed.

We find in this experiment that 0·02–0·1 % quinine hydrochloride has a marked accelerating effect on urease, as I supposed. But if I continued this experiment, I should no longer find this effect, because the free quinine base is toxic for urease. Table XVIII shows us this fact. Quinine base is difficult to dissolve in water. Therefore 0·06 g. of quinine was dissolved at first in about 2 cc. of absolute alcohol and was diluted with distilled water to 100 cc. Such a small quantity of ethyl alcohol has no effect on urease as is shown later on.

TABLE XVIII.

H_2O in cc.	0·06 % quinine in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 22 hrs in cc.
40	—	5	5	6·84
—	40	,,	,,	2·87
20	20	,,	,,	4·01
30	10	,,	,,	4·65
35	5	,,	,,	5·01

It is evident that free quinine base inhibits urease action apparently even in a concentration of 0·006 %. This effect is not due to the hydroxyl ion

because 0·006 % quinine is only about mol./6300; it is apparently the effect of the alkaloid ion.

Laqueur [1906] examined the effect of quinine hydrochloride on various ferments. In accordance with many other workers he observed that quinine hydrochloride, generally speaking, inhibited enzymes, but sometimes in its diluted concentration accelerated the ferment action. As indicated by Traube and the author [1914], and as the present work suggests, it is desirable to distinguish between the results obtained by quinine salt and quinine base. It would be wrong to ascribe the effect of "quinine salts" to an effect of "quinine," as is sometimes done.

The same inhibitory results were observed with free atropine and pilocarpine base. Mixing of atropine and pilocarpine had only an additive effect but not an antagonistic effect on urease. The experiments of this section show that the effects of neutral salts are due to the alkalinity of metal bases and alkaloid bases to a certain limit. They do not agree with van Slyke's results concerning *c* and *d*.

(4) *Effects of alcohols on urease.*

Armstrong, Marshall, and van Slyke reported the retarding effect of alcohols on urease. Armstrong showed that alcohols which have less solubility retard the urease action more strongly than alcohols with greater solubility. Van Slyke stated that ethyl alcohol in 30 volumes per cent. (5·7 mol.) retarded both *c* and *d*, especially the latter. He ascribed this effect to the high osmotic pressure of the alcohol solution. He stated that a 2 M solution of alcohol is the limiting concentration for retardation of *d* as well as of *c*.

Alcohols are substances which are able to lower the surface tension of water considerably and we may therefore expect here some considerable

TABLE XIX.

H_2O in cc.	5 M alcohol in cc.	0·5 % urease in cc.	1·5 % urea in cc.	Conc. of alc. in M	N/10 NH_3 developed in 5 hrs in cc.	
20	—	5	5	—	9·43	9·41
,,	—	,,	,,	—	9·39	
—	Methyl 20	,,	,,	3·3	8·75	8·84
—	,,	,,	,,	,,	8·93	
—	Ethyl 20	,,	,,	,,	7·65	7·30
—	,,	,,	,,	,,	6·95	
—	Propyl 20	,,	,,	,,	7·62	7·31
—	,,	,,	,,	,,	7·00	
—	M/8 amyl 20	,,	,,	1/12	8·80	8·78
—	,,	,,	,,	,,	8·76	
30	M/2 paraldehyde 20	,,	,,	1/6		1·15

effects of strongly active substances, like alcohols, on urease. Can it be justifiable to take into consideration here only the osmotic pressure-capacity factor of osmosis of Traube?

My experiments were directed at first to confirm the results obtained by others. Concentrated alcohol solutions were employed (Table XIX).

Addition of paraldehyde caused immediate precipitation of urease solution.

We see from this table an obvious depressing effect of methyl, ethyl, and propyl alcohol in 3·3 M and amyl alcohol in M/12. Paraldehyde in M/6 inhibited urease considerably.

This order of alcohols in relation to their depressing effect is the order of decrease of solubility, surface-tension activity and "attraction pressure" of Traube. The reason why the difference of effect between ethyl and propyl alcohol is not so distinct as is expected from the surface-tension activity, may be the too high concentration of alcohols used, or the time during which the digestion bottles were left at room temperature which was not suitable for the observation of this difference (see later).

Experiments were next carried out to examine the effect of diluted alcohols. The results were contradictory to the reports of previous investigators. In order to attain certainty the experiments were repeated carefully.

TABLE XX *A*.

Concentration of alcohol	N/10 NH_3 developed in 18 hours in cc.
0·5 % urease alone	23·52
M methyl	23·63
M ethyl	23·87
M/10 amyl	23·87
M/16 amyl	23·83

TABLE XX *B*.

Concentration of alcohol	N/10 NH_3 developed in 7 hours in cc.
0·5 % urease alone	6·66
M methyl	7·69
M ethyl	7·98
M/10 amyl	8·09

TABLE XX *C*.

Concentration of alcohol	N/10 NH_3 developed in 6·7 hours in cc.	
0·5 % urease alone	7·25 7·24	7·25
M methyl	8·86 8·97	8·92
M ethyl	8·75 8·55	8·65
M propyl	8·53 8·75	8·64
M/20 amyl	8·50 8·34	8·42

Alcohols in diluted condition accelerate the urease action. It is beyond question; methyl, ethyl, and propyl alcohol in a concentration of 1 M and amyl alcohol in one of M/20 per litre.

Another experiment with more dilute alcohols was carried out.

TABLE XXI.

Concentration of alcohol	$N/10$ NH_3 developed in 7 hours in cc.	
0·5 % urease alone	6·00	5·99
" "	5·97	
M/5 methyl	5·97	6·08
"	6·18	
M/5 ethyl		6·06
M/5 propyl	5·90	6·21
"	6·51	
M/40 amyl	6·47	6·46
"	6·44	

It is evident that methyl, ethyl and propyl alcohol in M/5 had only a feeble accelerating effect, but M/40 amyl alcohol still had a somewhat more recognisable accelerating action.

It is necessary to notice here that, in order to find the accelerating or retarding effect of some substance on ferments, it is very important that the conditions of duration of ferment action and relative concentration between ferment and substrate must be carefully adjusted.

In Table XX *A* we cannot see distinctly the accelerating effect of alcohol. The reason for this is the longer duration of the digestion, so that the process of hydrolysis went on nearly to the end, both with and without the addition of alcohols. On the other hand, to confirm the above assumption, I have experimented with relatively concentrated alcohol solutions. In this case the concentration of alcohol was the same as in Table XX—1 M, but the urease solution was relatively less concentrated, 0·4 % being used instead of 0·5 %. The digestion bottles were also left a little longer at room temperature.

TABLE XXII.

Concentration of alcohol per litre	$N/10$ NH_3 developed in 22 hours in cc.	
0·4 % urease alone	22·99	22·90
" "	22·80	
M methyl	22·78	22·72
"	22·66	
M ethyl	22·63	22·67
"	22·71	
M propyl		22·72
M butyl	22·79	22·77
"	22·76	
M/40 amyl	22·80	22·80
"	22·80	

As is seen, there was a slight depressing effect produced by 1 M solutions of the alcohols on urease. That is quite contradictory to the result where this same concentration of the alcohols was able to accelerate the urease action.

Provided that the conditions mentioned above are fulfilled, we learn that concentrated alcohols inhibit urease, while diluted alcohols, on the contrary, accelerate its action.

It is probable that many previous investigators did not succeed in observing this accelerating effect of alcohol on the urease action, because the conditions mentioned above were not satisfied. The alcohol solutions may have been absolutely or relatively too concentrated or the duration of urease action too short.

There are still some points of interest to be considered. It is strange that here alcohols facilitate the enzyme action, while we are taught that alcohols usually paralyse various chemical and biological reactions. My result however is not the only one of the kind, since Giselt [1905] has observed the accelerating effect of alcohol on the action of pancreatic lipase.

Now again, the question arises: To what properties of alcohols are their inhibitory and accelerating effects on urease due?

If we try to explain these facts only by osmotic pressure, then it is rather curious that one property can act in two contrary directions.

Traube alleged long since that osmotic pressure is only a capacity factor of the mechanics of osmosis—number of the dissolved particles—and another important driving factor of osmosis—intensity factor—is neglected. Traube called the latter attraction pressure or "Haftdruck." The substance which has less attraction pressure against water accumulates on the surface of the second phase in contact with water, and lowers the surface tension of water. This fact has long been known as Gibbs' theorem and has been experimentally proved.

We do not therefore exhaust all the possibilities when we take into consideration only the osmotic pressure in dealing with substances which have a large effect on surface-tension and with colloidal particles having immense surface development.

With regard to the cause of the accelerating effect of dilute alcohols on urease action there are two possibilities in reference to the considerations mentioned above.

1. Alcohol molecules carry urea molecules with them together to the surface of urease particles, when alcohol molecules accumulate on the surface of enzyme. This process facilitates the contact between enzyme and substrate.

2. Change of surface-tension of urease solution disperses the urease particles and thus increases their active surface.

With regard to the depressing effect of concentrated alcohols on urease there are also two possible causes.

1. Alcohol molecules accumulating on the surface of enzyme particles disturb the contact between urease particles and urea molecules or drive off urea molecules from the surface of enzyme particles.

2. Enormous change of surface-tension favours aggregation of enzyme particles.

Supposing that the first consideration might be the case, I planned my experiment as below.

We learned that formic acid had a moderate inhibitory effect on urease. If we add urease to a mixture of alcohol and formic acid we may expect the inhibitory effect of formic acid to be increased by dilute alcohol, and we may expect with the concentrated alcohol, on the contrary, the diminution of the inhibitory effect of formic acid.

Formic acid was mixed and shaken with ethyl alcohol, and water and 0·5 % urease were then added. In the whole mixture the formic acid was N/2000 and alcohol was 1 and 3·3 molar respectively. The acid was allowed to act on the urease for 15 minutes, and was then neutralised. Urea was added, each bottle was brought to an equal quantity of content and left for 22 hours at room temperature.

TABLE XXIII.

H_2O in cc.	N/200 formic acid in cc.	5 M ethyl alcohol in cc.	0·5 % urease in cc.	Concentration of alcohol M	neutralised by addition of	M/200 NaOH in cc.	1·5 % urea in cc.	H_2O in cc.	N/10 NH_3 developed in 22 hrs in cc.	
40	—	—	5	—		—	5	5	23·18	
35	5	—	,,	—		5	,,	—	10·35	10·6
,,	,,	—	,,	—		,,	,,	—	9·76	
25	,,	10	,,	1		,,	,,	—	10·14	10·15
,,	,,	,,	,,	,,		,,	,,	—	10·16	
2	,,	33	,,	3·3		,,	,,	—	13·08	12·54
,,	,,	,,	,,	,,		,,	,,	—	11·99	

The bottles with 1 M ethyl alcohol and acid developed practically as much ammonia as the bottles with acid alone. But if we consider that 1 M ethyl alcohol is able to accelerate the urease action we come necessarily to the conclusion that 1 M ethyl alcohol facilitated the inhibitory effect of formic acid. In the case of 3·3 M ethyl alcohol we may expect the additional result of the inhibitory effect of acid and the depressing effect of concentrated alcohol if we do not take into consideration the influence of the change of

surface-tension. In fact, we find in this experiment a distinct increase of ammonia in the bottles with acid and 3·3 M ethyl alcohol. It shows us that the action of the urease particles has been relieved from acid inhibition by concentrated alcohol. It has over-compensated the loss caused by the depressing effect of concentrated alcohol.

Let us consider the second possibility mentioned above. If the effect of 1 M alcohol was merely in the dispersion of enzyme particles, but not in facilitating the contact between substrate and urease particles, we must have a larger yield of ammonia from the bottles with acid and 1 M ethyl alcohol than from the bottles with acid alone. But this was not the case.

For the same reason, if 3·3 M alcohol had no effect in driving off acid from the surface of urease particles, but only the aggregating effect on urease, we must get a smaller yield of ammonia from the bottles with 3·3 M ethyl alcohol and acid than from the bottles with acid alone. But this was also not the case.

The question next arises, Does not alcohol combine with formic acid to deprive the latter of its inhibitory effect?

As compared with the concentration of N/2000 formic acid, 1 and 3·3 M ethyl alcohol are very concentrated. If the process concerned is only ester formation between alcohol and formic acid, then 1 and 3·3 M alcohol are strong enough to combine with all the formic acid. We may expect then in Table XXIII only the accelerating effect of diluted alcohol and the retarding effect of concentrated alcohol. But this also was not the case, the result was the reverse.

Further, if the effect of dilute alcohol is in the facilitation of combination between urease and urea, and the effect of concentrated alcohol is in the driving off urea from the surface of urease particles, we can assume as follows. If concentrated urea solution is used as substrate, then the combination of urea molecules with urease particles will be so easy that addition of 1 M alcohol has no effect. On the other hand in such conditions urease particles will be surrounded by so many urea molecules that addition of concentrated alcohol will not be able to drive off the urea molecules from the surface of urease particles in order to take their place.

For this purpose I used 5 % urea solution instead of the usual 0·15 %.

This result proves the above assumption to be true. 3·3 M ethyl alcohol even accelerated the urease action a little. Compared with the high concentration of urea, 3·3 M ethyl alcohol is a relatively diluted solution, so the driving power of alcohol is not stronger than the adhering power of

TABLE XXIV.

H_2O in cc.	5 M ethyl alcohol in cc.	0·5 % urease in cc.	25 % urea in cc.	Concentration of alcohol M	N/10 NH_3 developed in 7 hours in cc.	
35	—	5	10	—	7·14	7·20
,,	—	,,	,,	—	7·25	
25	10	,,	,,	1	7·60	7·40
,,	,,	,,	,,	,,	7·19	
2	33	,,	,,	3·3	7·53	7·72
,,	,,	,,	,,	,,	7·90	

urea to urease. This must be the reason of the acceleration. 1 M ethyl alcohol accelerated only a little. These results teach us also that the effect of alcohol is not in a dispersion or aggregation of urease particles.

There is still one more question. According to Traube the lowering of surface-tension by methyl, ethyl, propyl, butyl and amyl alcohol increases in the proportion of $1:3:3^2:3^3:3^4$. But in my series of experiments I could not see such enormous differences in the effect of these alcohols. I am of opinion that this was due entirely to unsatisfactory experimental conditions. I tried, therefore, to resolve this question from the other side. I prepared isocapillary alcohol solutions and examined their effect on urease.

Traube's stalagmometer was used for this purpose. It contained 56·9 drops of water at 20°; 5 M methyl alcohol showed 79·1 drops. The number of drops of alcohol solutions and the data of the experiments are as follows. The dilution of alcohols to make isocapillary solutions corresponded nearly to the law of Traube mentioned above.

TABLE XXV.

H_2O in cc.	10 cc. of alcohols: Name	10 cc. of alcohols: No. of drops water	1·5 % urea in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 6·7 hours in cc.	
40	—	56·9	5	5	14·30	14·23
,,	—	,,	,,	,,	14·16	
30	Methyl	79·1	,,	,,	15·46	15·48
,,	,,	,,	,,	,,	15·50	
,,	Ethyl	79·0	,,	,,	14·74	15·09
,,	,,	,,	,,	,,	15·44	
,,	Propyl	78·9	,,	,,	14·62	14·45
,,	,,	,,	,,	,,	14·28	
,,	Butyl	79·0	,,	,,	14·68	14·78
,,	,,	,,	,,	,,	14·87	
,,	Amyl	78·9	,,	,,	14·61	14·67
,,	,,	,,	,,	,,	14·72	

The results were quite decisive. All the alcohols employed accelerated nearly in the same degree. The alcohol solutions were not quite isocapillary but the number of drops of these solutions varied between 78·9 and 79·1.

The results obtained were very instructive, because the yield of ammonia was exactly proportional to the number of drops, i.e. to the surface-tension. A difference of 0·1 drop in nearly 80 drops showed itself distinctly in the effect on urease action.

It is necessary to emphasise once more the fact that, in the discussion of the effect of substances, which have a large effect on surface-tension, on ferments, warning must be given earnestly against taking only osmotic pressure into consideration; another important factor, surface-tension, must be remembered. The present series of experiments proved this idea to be quite justified.

(5) *On the diminution of action of urease during preservation.*

Traube and the author [1914] proved by means of dark ground illumination that the diminution of toxicity of atropine in a watery solution during preservation is to be ascribed to the aggregation of colloidal particles of atropine. We supposed that this process would follow the formula of unimolecular reactions and in fact we found the (k) of this process fairly constant.

Van Eck [1911] found that the destruction of peroxidase in milk by heat proceeds as a reaction of the first order and the (k), obtained by using the formula of a unimolecular reaction, was constant, and this was confirmed by Zilva [1914].

It is not without interest, therefore, to investigate urease in this direction.

1 % urease solution was kept under toluene in a room where the change of temperature was not great. The bottle with urease was exposed to diffuse light. During 52 days' preservation the efficiency of the urease was estimated at intervals.

5 cc. of the urease solution were mixed with 5 cc. of 1·5 % urea and brought up to 50 cc. with distilled water. The digestion bottle was kept in the water bath at 16° for 7 hours; then the urease action was checked and ammonia was estimated.

During preservation some amount of precipitation took place.

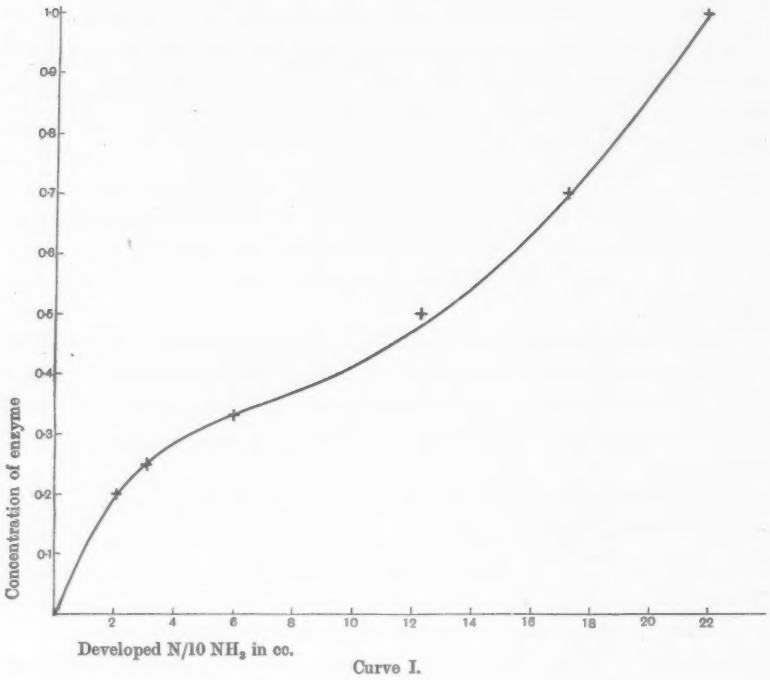
In order to find out the relation between the concentration of urease and the development of ammonia by urease I performed the following experiment.

At the start of this experiment out of the above-mentioned 1 % urease solution 5, 3·5, 2·5, 1·67, 1·25 and 1·0 cc. had been measured and the amount of NH_3 developed by these quantities of urease under the same conditions as stated above was determined. The relation of urease concentration and yield of ammonia was the following:

TABLE XXVI.

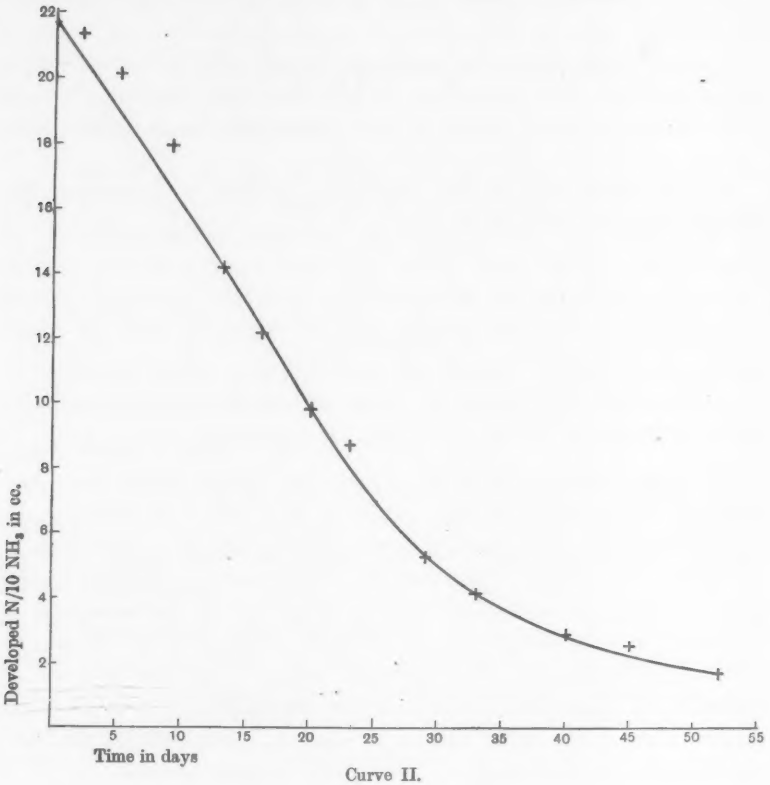
Concentration of enzyme	N/10 NH_3 developed in cc.
1·0 (5 cc. of 1 % urease)	21·65
0·7	17·17
0·5	12·20
0·33	5·96
0·25	3·01
0·2	2·02

These data are shown graphically in Curve I.



Curve I.

The following curve and table show the amount of ammonia developed by preserved urease and the calculated concentration of urease on the various days of experiment. This calculation was carried out by making use of Curve I and the quantity of developed ammonia.



Curve II.

TABLE XXVII.

Days of preservation	N/10 NH_3 developed in cc.	Calculated concentration of urease		
$t=0$	21·65	1·00 $=a$		k
2	21·37	0·97		0·0152
5	20·10	0·88		0·0256
9	17·96	0·74		0·0334
13	14·16	0·55		0·0460
16	12·16	0·48		0·0459
20	9·80	0·41	$a-x$	0·0446
23	8·71	0·38		0·0421
29	5·26	0·32		0·0465
33	4·10	0·29		0·0375
40	2·90	0·25		0·0346
44	2·55	0·23		0·0333
52	1·72	0·17		0·0341

If a unimolecular reaction is not reversible we have the well-known formula:

$$k = \frac{1}{t} 2{\cdot}302 \log \frac{a}{a-x}.$$

I took here 24 hours as a unit of t and obtained k as shown above.

A steady change of k was here observed; it reached its maximum on the 29th day and then began to fall.

Summary.

1. The effect of various acids, alkalis, alcohols, aldehyde, neutral salts and alkaloids on the urease of soy-bean has been studied.

2. In the inhibitory effects of inorganic and organic acids on urease the hydrogen ion concentration plays the main rôle. These inhibitory effects are influenced by the physical properties, and especially the surface-tension, which the acids possess. Total inhibitory effects are considered to be the combined effects of chemical and physical properties. If the dissociation of the acids is equally strong, then the inhibitory effects of acids with greater effect on surface-tension obtain the upper hand. Even the deficiency of hydrogen ion concentration can be over-compensated by great effect on surface-tension. The acids with great effect on surface-tension facilitate the adsorption of hydrogen ions on the surface of urease particles.

3. The inhibitory effects of caustic soda and ammonia do not coincide in reference to the hydroxyl ion concentration. Soda inhibited urease in far lower concentration than ammonia. But in equally effective soda and ammonia solutions the hydroxyl ion concentration is far greater in soda solution. The inhibitory effect of soda is to be ascribed to the hydroxyl ion concentration but ammonia has some further action.

4. Provided that certain conditions are fulfilled, it has been proved that 1 M alcohols (methyl, ethyl, propyl) and M/40 amyl alcohol accelerate the urease action, but 3·3 M of the former group and M/12 of the latter retard the urease action.

5. Isocapillary alcohol solutions manifest equal effects on urease.

6. The cause of this acceleration or retardation is not to be understood merely in the light of osmotic pressure—Traube's capacity factor of the mechanism of osmosis. The intervention of Traube's intensity factor—attraction pressure, represented here by the surface-tension—makes clear the explanation of these phenomena.

7. The dilute alcohols which lower the surface-tension of the solvent accumulate on the surface of the second phase in contact with the solvent. By this action they facilitate adsorption between urease and urea. This is probably the cause of the acceleration of the urease action. Concentrated alcohols disturb the adsorption between urease and urea by displacement. This is the cause of retardation of the urease action.

8. In this work it is not found that the effects of alcohols on urease were caused by the dispersion or aggregation of urease particles.

9. Aldehyde inhibits urease notably.

10. The effects of neutral salts are merely those of their metallic bases. They retard in N/10 strength the urease action, because metallic bases displaced by ammonia produce a greater hydroxyl ion concentration than the equivalent of ammonia.

11. In accordance with this, alkaloid salts (quinine is a weaker base than ammonia) accelerate urease action in the first stage of hydrolysis. But alkaloid bases themselves inhibit urease action markedly (quinine, atropine and pilocarpine). The effects of the atropine and the pilocarpine on the urease are additive but not antagonistic.

In conclusion, I have pleasure in expressing my thanks to Prof. Starling, Prof. Bayliss and Dr Plimmer, for their kindness in allowing me to work in their institute and for their advice. The theme of this work was suggested by Prof. Bayliss and I worked under his supervision.

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LI. ON THE UREASE OF THE SOY-BEAN AND ITS "CO-ENZYME."

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(*Received November 15th, 1915.*)

In 1897 Bertrand introduced the term "co-enzyme." Since that time, co-enzymes have been discovered for various enzymes. The mode of action of co-enzymes is not yet quite clear, but the constitution of some co-enzymes is definitely determined. For instance, hydrogen ion for pepsin, hydroxyl ion for trypsin, electronegative ions such as chlorine and bromine ions for pancreatic amylase (Henri) and bile-salts for the lipase of liver (Magnus); [for literature see Bayliss, 1914, p. 123].

The constitution of other co-enzymes is not yet known and it is likely that these co-enzymes consist of a complicated system of several components. Harden and Young's [1906, 1908, 1910] co-enzyme of the zymase of yeast juice consists apparently of unknown components. Recently Neuberg and Schwenk [1915] found that salts of 2-ketonic acids act on yeast juice or dried yeast like the co-enzyme, but some important factor is wanting since the effect of salts of 2-ketonic acids is not as strong as that of the natural co-enzyme.

The "co-enzyme" of urease belongs also to the group of those with unknown constitution. Indeed, its nature, as will be seen, may throw some doubt on its claim to the title.

Experimental.

Methods employed.

The method employed for the estimation of ammonia which was produced by urease action was the same as that described in the previous paper [Onodera, 1915]. Only a few words may be added. Precipitated urease prepared according to van Slyke was used as the source of enzyme. The contents of each digestion bottle were always 50 cc. and that always included

5 cc. of 1·5 % urea solution. This amount of urea corresponds to 25 cc. of N/10 NH_3. The digestion bottles were left in a water bath at room temperature.

1. *Urease loses its activity by dialysis.*

Dialysis was always carried out at room temperature by means of parchment paper, the urease solution being covered with toluene. Distilled water was used as a dialysate and it was either changed at intervals or continuously by means of a syphon. Dialysis was stopped when the chloride reaction of the dialysate was negative. The loss of activity of the enzyme by dialysis is due either to change of enzyme sustained during the course of dialysis or to the passing of enzyme particles through the parchment paper or to the loss of co-enzyme.

Loss of activity of enzyme owing to preservation is excluded since the control undialysed urease preserved under toluene was always very active (see below).

Urease is capable of passing to some extent through thin parchment paper, but not through the parchment paper employed in these experiments. Moreover an experiment is recorded in section 4 showing that this did not take place. It appears, therefore, that some essential constituent was removed from the urease by dialysis. This constituent may, for brevity, be called "co-enzyme."

In the following sections, several experiments are described proving that assumption.

2. *A small amount of fresh urease is able to revive the lost activity of the dialysed urease.*

TABLE I.

1·5 % urease dialysed for 5 days in cc.	0·5 % fresh urease added in cc.	N/10 NH_3 developed in 16·5 hours in cc.
5	—	0·08
5	0·5	4·62
5	1·0	8·53
—	1·5	4·03
—	1·0	1·23
—	0·5	0·15
Control urease not dialysed, 3 times diluted		
5	—	23·19

As is seen in this table, 1·5 % urease lost its activity by 5 days' dialysis, and 5 cc. of this dialysed urease developed only 0·08 cc. of N/10 NH_3, while 5 cc. of the control urease, even three times diluted, hydrolysed almost all the urea present in nearly the same time. The addition of 0·5 and 1·0 cc. of 0·5 % fresh urease revived the lost action markedly, while the sum of the ammonia produced by the dialysed and the fresh urease each separately was much less.

The question arises whether this reviving action of the fresh urease is not an illusory phenomenon and whether it is not a simple additional effect of fresh urease on the urease which might be diluted by dialysis. The curve of urease concentration and ammonia development given in the previous paper [Onodera, 1915, p. 570] shows that the amount of ammonia produced by the quantity $(a + b)$ cc. of urease is greater than the sum of ammonia which is produced by a cc. and b cc. of urease respectively. From this curve we obtained the following data:

0·5 % urease in cc.	N/10 NH_3 developed in cc.
0·5	1·0
1·0	2·0
1·5	4·6
2·0	9·4
2·5	12·8
3·0	15·2
3·5	17·2
4·0	18·8

Making use of these data, if we suppose that a certain quantity of the dialysed urease can produce 1·0 cc. N/10 NH_3, then that quantity corresponds exactly with 0·5 cc. of 0·5 % urease. Now suppose that we add to that dialysed urease 1 cc. and 1·5 cc. of 0·5 % fresh urease respectively. By the simple addition of the corresponding quantities of ammonia we get 3 cc. and 5·6 cc. of N/10 NH_3 respectively. But in fact 1·5 and 2·0 cc. of 0·5 % fresh urease produced 4·6 and 9·4 cc. respectively.

The increase of the amount of ammonia produced by the further addition of urease, as is seen in the previous data, is not so great as it is in Table I.

In the former case, the increase of yield of ammonia does not exceed twice the arithmetic sum of the separate amounts of ammonia. In the latter case, the increase reaches to ten or twenty fold. According to these considerations, the presence of co-enzyme in the fresh urease and the loss of co-enzyme by dialysis was definitely settled.

3. *Is this co-enzyme an inorganic salt and is it soluble in ether?*

A certain volume of 1 % urease was dried, incinerated and was dissolved in a quantity of distilled water equal to its original volume. 5 cc. of 1·5 % urease, which was dialysed for 5 days, developed 0·13 cc. of N/10 NH_3 in 16·5 hours. The same quantity of the dialysed urease with 5 cc. of the solution of the ash developed only 0·22 cc. of N/10 NH_3 during the same time.

Rosenheim's co-enzyme or activator of pancreatic lipase could be extracted from the ash only by means of hydrochloric acid [Pekelharing, 1912]. I tried this procedure also with urease. Urease was incinerated with hydrochloric acid and the ash was dissolved in water. 5 cc. of the same dialysed urease developed 0·33 cc. of N/10 NH_3 in 20 hours while an equal quantity of the dialysed urease with the solution of the ash produced only 0·36 cc. of N/10 NH_3.

20 cc. of 1 % urease were shaken with 50 cc. of ether and the ether separated. After evaporation of the ether the residue was suspended in 20 cc. of distilled water. 15 cc. of this solution mixed with 5 cc. of the dialysed urease developed 0·33 cc. of N/10 NH_3 in 20 hours, while the dialysed urease alone produced 0·36 cc. of N/10 NH_3 during the same time. I tried to ascertain whether the co-enzyme was in the original solution. The urease solution after ether extraction was freed from ether by drawing an air current through it, the aeration being continued nearly half an hour until the smell of ether disappeared. With this solution the following experiment was made.

TABLE II.

Dialysed urease in cc.	1 % urease after extraction with ether	N/10 NH_3 developed in 17·7 hours in cc.
5	—	0·67
—	5	1·84
5	5	2·62

It will be seen that the urease action was inhibited very strongly and no activator was found in it. We can judge from this fact that ether destroyed the co-enzyme and that it could not be restored by the driving away of ether.

The dialysed urease employed in this series of experiments was, of course, reactivated when fresh urease was added.

We can conclude that the co-enzyme is not an inorganic salt and does not pass into ether but is destroyed by ether.

4. *Can the co-enzyme be found in the dialysate?*

For the purpose of solving this question, the following experiment was carried out. 0·5 % urease was subjected to dialysis for 4 days, the amount of dialysate being 5 times as great as the urease. The dialysate was not changed. After dialysis the dialysed urease solution was found in the same quantity as at starting. During dialysis both the urease solution and the dialysate were covered by toluene. The data obtained with these materials are given in Table III.

TABLE III.

Urease in cc.		N/10 NH_3 developed in 16·3 hours in cc.
0·5 % urease not dialysed	5·0	15·70
0·5 % urease dialysed	5·0	0·11
Dialysate	25·0	− 0·06
0·5 % urease dialysed Dialysate	5·0 25·0	0·22

By this experiment it was proved that the urease inactivated by dialysis could not be reactivated by the addition of dialysate. It was moreover shown that no urease particles passed through the parchment paper. This dialysed urease solution recovered its activity considerably when co-enzyme was added, as the following table shows.

TABLE IV.

Dialysed solution in cc.		0·5 % fresh urease in cc.	N/10 NH_3 developed in 16·5 hours in cc.
0·5 % urease not dialysed, as control	2·5	—	3·36
0·5 % urease dialysed ...	2·5	—	0·08
Dialysate	12·5		
0·5 % urease dialysed ...	2·5	1·0	7·70
Dialysate	12·5		
—		1·0	0·60

Co-enzyme was lost by dialysis but it could not be detected in the dialysate. What change then might have occurred to the urease during dialysis?

(*a*) Is co-enzyme dialysable but destroyed immediately after passing through parchment paper into the dialysate?

(*b*) Is co-enzyme not dialysable but changed into an inactive state through the diminution of salts owing to dialysis? It is possible that the precipitation of proteins may have been the principal cause. In fact, notable precipitation occurred in the urease solution during dialysis, while the control urease solution remained unchanged.

(*c*) Is co-enzyme adsorbed by the dialysing parchment paper?

No definite ground could be found for answering the first question with confidence. It is however very unlikely.

To answer the second question, the following experiments were carried out. After dialysis, the dialysed urease solution was filtered and the precipitate was suspended in distilled water, the volume of the suspension being made equal to that of the filtered urease. (To avoid confusion I use in the following description the abbreviations (d.f.) for the filtrate and (d.p.) for the suspension of the precipitate from the dialysed urease.) With d.f. and d.p. experiments were carried out.

TABLE V.

1·5 % urease dialysed for 5 days in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 17 hours in cc.
d.f. 5	—	0·95
d.p. 5	—	0·88
d.f. 5 d.p. 5	—	3·07
d.f. 5	2·0	16·05
d.p. 5	2·0	8·70
—	2·0	2·00
1·5 % urease not dialysed, as control 5	—	22·66

TABLE VI.

0·5 % urease dialysed in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 17 hours in cc.
d.f. 5	—	0·01
d.p. 5	—	0·23
d.f. 5	2·0	7·52
d.p. 5	2·0	5·62
—	2·0	1·70

In Table V we see that d.f. and d.p. were not able to activate each other. It indicates that co-enzyme was neither in the solution nor in the precipitate of the dialysed urease.

If we compare the activities of d.f. and d.p. alone, we do not find any distinct difference in Table V but in Table VI d.p. was a little stronger than d.f. When d.f. and d.p. had been activated by addition of fresh urease they showed a distinct difference. The activated d.f. developed markedly more ammonia than the activated d.p. The increased development of ammonia in the activated d.p. gives us no proof that the increase was chiefly to be ascribed to the activating effect of co-enzyme added (fresh urease).

From the data of Tables V and VI we may conclude that inactivated enzyme was present in the filtrate d.f. The precipitate d.p. contained a

smaller quantity of enzyme proper than the filtrate, but still, although very small, some quantity of co-enzyme. This co-enzyme might possibly be supposed to be fixed by the enzyme. For this reason, the precipitate, in spite of its activity, could not activate the filtrate. From these facts I distinguish two kinds of co-enzyme of urease. One is the fixed co-enzyme and the other is the free co-enzyme.

In the following experiments it is clearly seen that the free co-enzyme has less resistance to various modes of treatment (such as dialysis, influence of heat, acid and alkali) than the fixed one. This fact agreed also with an experiment on the germination of soy-bean (see later).

Some reactions of the precipitate were studied. 1·5 % urease was subjected to dialysis for 4 days. The precipitate was collected and was made up with distilled water to a suspension equal in volume to the original solution. The dialysis was not quite sufficient, consequently the dialysed urease still showed some activity. The suspension was like milk and quite neutral. The addition of two drops of saturated $MgSO_4$ solution, 0·2 cc. of N/10 NaOH, or four drops of N/10 H_2SO_4 to 2 cc. of the suspension cleared away the milky state. This reaction was interesting, remembering that urease solution always becomes turbid when it is mixed even with a very weak acid. $CaCl_2$ had no effect. The suspension showed a distinct biuret-reaction. An experiment on digestion of the precipitate was made. 5 cc. of 1 % trypsin were added to 20 cc. of the suspension. The mixture was subjected to digestion at 37° for 2 days and was then dialysed for 2 days; the biuret-reaction was negative in this dialysed mixture.

An experiment was then made with the mixture.

10 cc. of the digested and then dialysed suspension developed 4·75 cc. of N/10 NH_3 in 18 hours.

5 cc. of 1 % trypsin developed 0·27 cc. of N/10 NH_3.

10 cc. of the suspension alone developed 7·42 cc. of N/10 NH_3 in 21 hours.

From this we learn that the urease was not destroyed by trypsin digestion. Van Slyke also found that urease resisted trypsin digestion.

I tried also to restore the co-enzyme from the precipitate, supposing this precipitation to be reversible. The precipitate was dissolved in a small quantity of NaOH, H_2SO_4, or $MgSO_4$ as described above and was added to dialysed urease, but these experiments all failed to restore the co-enzyme.

It seems very likely that the disappearance of co-enzyme was due to precipitation during dialysis and that it remained in the dialysed solution in an inactive state, but I could not succeed in proving it.

As to the third question, the adsorption of co-enzyme by parchment paper was definitely excluded. The following experiment shows this.

5 cc. of dialysed urease developed 0·08 cc. of N/10 NH_3 in 16·4 hours.

5 cc. of dialysed urease with parchment paper cut in pieces developed 0·06 cc. of N/10 NH_3 in 16·4 hours.

5 cc. of dialysed urease + 1·0 cc. of 0·5 % fresh urease developed 8·53 cc. of N/10 NH_3 in 16·4 hours.

5. *Can co-enzyme be filtered through a Berkefeld porous filter?*

Rosenheim's co-enzyme of pancreatic lipase could easily be separated by filtration through Pukall's porous clay filter [Umeda, 1915]. The same experiment was therefore tried with urease.

After filtration, a white precipitate remained on the filter. The precipitate was collected and treated with a quantity of distilled water equal to that of the filtrate so as to make a suspension.

Investigations of the activity of the filtrate as well as of the suspension and of their contents of co-enzyme were carried out. The results obtained were as follows:

TABLE VII.

0·5 % urease dialysed in cc.	Urease treated with clay filter in cc.		N/10 NH_3 developed in 17·25 hours in cc.
	Filtrate	Residue	
5	—	—	0·13
5	2	—	2·48
5	—	2	3·92
—	2	—	0·68
—	—	2	0·80

TABLE VIII.

0·5 % urease dialysed in cc.	Urease treated with clay filter in cc.		N/10 NH_3 developed in 18·7 hours in cc.
	Filtrate	Residue	
5	—	—	0·08
5	1	—	3·82
5	—	1	1·27
—	1	—	0·62
—	—	1	0·09
—	5	5	22·07

As is seen in Tables VII and VIII, the activities of the filtrate and the residue were not constant; in the case of Table VIII the activity of the residue was very small. This difference was caused probably by the mode of preparing the urease solution and the conditions at filtration, but it was shown that

no urease was lost by filtration through a porous filter. As Table VIII shows, the mixture of filtrate and residue in corresponding proportions had a very strong activity.

Further, the filtrate and the residue both contained co-enzyme. Their activity went quite parallel to their contents of co-enzyme. It was therefore clear that in the case of urease the enzyme proper and the co-enzyme both passed through the porous filter.

6. *Investigation of the distribution of urease in soy-bean or in its germ.*

It is known that some enzymes are distributed only in certain parts of plants, cells, etc. The distribution of the enzyme itself and of its co-enzyme might be different, so that the following experiment was made.

I separated the shell of the soy-bean from the bean substance or kernel and made a 1 % water extract from each of these two parts, with which experiments were made.

TABLE IX.

1·5 % urease dialysed in cc.	1 % extract of bean-shells in cc.	1 % extract of bean-kernel in cc.	N/10 NH_3 developed in 16·4 hours in cc.
5	—	—	0·45
5	2	—	0·50
5	—	2	1·73
—	2	—	−0·01
—	—	2	0·22

We learn from these data that the shell contained neither urease nor co-enzyme while the bean substance contained both.

I investigated then whether germination of the soy-bean was able to change the distribution of enzyme and co-enzyme.

Soy-beans were washed with distilled water and were arranged in rows on sheets of filter paper and were then covered again by several sheets of filter paper. They were all kept in glass vessels and moistened with distilled water. After keeping them in darkness for three days, the commencement of the growth of the germs was noticed. Here the germs, shell and proper substance were separated from each other. 1 % water extracts were prepared from each of the three parts and the following experiment made:

TABLE X.

5 cc. of 1 % germ-extract	developed	17·34	cc. of N/10 NH_3	in	18·2	hrs.
„ kernel-extract	„	9·42	„	„	„	„
„ shell-extract	„	0·18	„	„	„	„

The weights of dry substance of the germs and the bean substance proper were 10·47 and 38·79 % respectively. Consequently, a 1 % watery extract of them nearly corresponded to 0·1 and 0·4 % respectively referred to the dried substances.

When we look at Table X with this knowledge, we notice immediately that the content of active urease in soy-bean germs must be very high. It is higher than in the precipitated urease itself. In contrast to this fact the absence of activating power on dialysed urease interested me very much (see Table XI).

TABLE XI.

1·5 % urease dialysed in cc.	1 % germ-extract in cc.	1 % kernel-extract in cc.	1 % shell-extract in cc.	N/10 NH_3 developed in cc.
5	—	—	—	0·13 in 16·5 hours
5	2	—	—	3·05 ,,
5	—	2	—	0·83 ,,
5	—	—	2	0 ,,
—	2	—	—	3·29 ,,
—	—	2	—	0·67 ,,
—	—	—	2	−0·02 ,,
5	—	—	—	0·04 in 17·7 hours
5	2	—	—	2·65 ,,
—	2	—	—	2·63 ,,

The process of germination of soy-bean is to be compared with dialysis, because in both processes the exchange of substances takes place between the urease or the bean-kernels and the surrounding water through a vegetable membrane. As a result, the co-enzyme of the germs and kernels was lost through the germination.

7. *Upon which constituent of urease, the diffusible or non-diffusible one, does acid or alkali act?*

An investigation was made to see which factor is influenced by these reagents. N/1000 HCl was allowed to act on urease for ten minutes, and was then neutralised by NaOH. Co-enzyme (fresh urease) and urease without co-enzyme (urease heated for an hour to 80° C., see following experiments) were added to the above-mentioned mixture and the activity measured with the result shown in Table XII.

It is to be noticed that the data concerning the inhibitory effects of various substances were not always uniform. Therefore in such cases I will describe the results of parallel experiments as obtained.

From this table one can easily see that N/1000 HCl only destroyed the diffusible part, and the remaining urease was put in action again by the

TABLE XII.

H_2O in cc.	0·5 % urease in cc.	N/200 HCl in cc.	Neutralised by addition of N/200 NaOH in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 20 hours in cc.	
45	5	—	—	12·5	—	5		22·10
35	5	10	10	2·5	—	5	0·10	0·07
35	5	10	10	2·5	—	5	0·05	
35	5	10	10	1·5	1	5	9·53	9·75
35	5	10	10	1·5	1	5	9·98	
35	5	10	10	0·5	2*	5		−0·02
40	—	10	10	0·5	2*	5		−0·03
40	—	10	10	1·5	1	5		0·20

* Heated to 80° C. for an hour.

addition of co-enzyme but not by the addition of the urease heated to 80° C. In a similar manner the effect of alkali was investigated. N/50 NaOH was allowed to act on urease for an hour.

TABLE XIII.

H_2O in cc.	0·5 % urease in cc.	N/10 NaOH in cc.	Neutralised by addition of N/10 H_2SO_4 in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 development in 27·7 hours in cc.	
45	5	—	—	11	—	5		22·52
35	5	10	10	1	—	5	7·17	6·85
35	5	10	10	1	—	5	6·53	
35	5	10	10	—	1	5	17·35	17·17
35	5	10	10	—	1	5	17·00	
40	—	10	10	—	1	5		0·74

From this table we may safely conclude that NaOH destroyed the greater part of the co-enzyme and this co-enzyme lost could be restored by the addition of fresh urease. I regret the data were not decisive, but those of Table XV supply this deficiency.

If acid and alkali act on co-enzyme only, we may assume that these substances will not be able to affect the dialysed urease, which is free from co-enzyme.

Experiments concerning this point are shown in Tables XIV and XV.

TABLE XIV.

H_2O in cc.	0·5 % urease dialysed in cc.	N/200 H_2SO_4 in cc.	Neutralised by addition of N/200 NaOH in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 18·7 hours in cc.	
45	5	—	—	11	—	5		0·16
35	5	10	10	1	—	5	0·00	0·00
35	5	10	10	1	—	5	0·01	
35	5	10	10	—	1	5	2·87	2·89
35	5	10	10	—	1	5	2·91	
40	—	10	10	—	1	5		0·29
45	5	—	—	10	1	5		3·00

TABLE XV.

H_2O in cc.	0·5 % urease dialysed in cc.	N/10 NaOH in cc.	Neutralised by addition of N/10 HCl in cc.	H_2O in cc.	0·5 % urease in cc.	1·5 % urea in cc.	N/10 NH_3 developed in 27·5 hours in cc.
35	5	10	10	1	—	5	0·03 } 0·02
35	5	10	10	1	—	5	0·01 }
35	5	10	10	—	1	5	3·41
40	—	10	10	—	1	5	0·71

From these tables we see that the previous hypothesis was confirmed. Dialysed urease itself was not inhibited by acid at all and could develop as much ammonia as the urease without the acid influence when fresh urease (co-enzyme) was added to them both. Acid and alkali therefore only destroy the co-enzyme but not the urease itself.

8. *What is the influence of heat on urease?*

Van Slyke and Cullen [1914] found that the optimum temperature for urease action was 55°; heating of urease to 60° for 30 minutes was ineffective, heating of urease to 70° for 30 minutes deprived it of 25 % of its activity, but heating to 80° for 30 minutes destroyed it completely. A first experiment was made with urease which was heated to 60° for half an hour.

TABLE XVI.

0·5 % urease heated to 60° for an hour in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 20·3 hours in cc.
5	—	22·31
5	1	22·28
—	1	0·44

TABLE XVII.

1·5 % urease dialysed for 5 days, in cc.	0·5 % fresh urea in cc.		N/10 NH_3 developed in 17·7 hours in cc.
	Not heated	Heated to 60° for half an hour	
5	—	—	0·04
5	2	—	12·32
5	—	2	10·76
—	2	—	1·54
—	—	2	1·57

In Table XVI we note that the urease was not injured at all by heating to 60° for an hour. The heated urease solution contained obviously a sufficient amount of urease proper and co-enzyme to hydrolyse the amount of urea present. Accordingly, the addition of a small amount of urease proper and co-enzyme did not show any appreciable effect.

But heating to 60° even for half an hour was not without effect on the urease system. As Table XVII clearly shows, some amount of the free co-enzyme was lost, and in regard to the capacity of reactivating dialysed urease the heated urease was inferior to the unheated.

Experiments were carried out with the urease which was heated to a higher temperature as follows:

TABLE XVIII.

0·5 % urease heated to 80° for an hour in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 20·3 hours in cc.
5	—	0·06
5	1	11·61
—	1	0·42

TABLE XIX.

0·5 % urease heated up to boiling point in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 17·25 hours in cc.
5	—	−0·02
5	1	10·93
—	1	0·43

It is clearly shown in these tables that heating to 80° for an hour or heating just up to boiling point totally destroyed the co-enzyme in the urease solution both fixed and free. But the addition of co-enzyme (fresh urease) enormously revived the action of the urease.

By these experiments the statements of van Slyke and Cullen as to the effect of heat on urease were confirmed. Further the heating of the urease solution above 80° totally destroyed the co-enzyme but not the urease proper. But the free co-enzyme was somewhat labile, it was already somewhat injured by heating to 60° for half an hour.

As a natural consequence it occurred to me that dialysed urease should not be destroyed by heating since it has no co-enzyme. An experiment to test this was made:

TABLE XX.

0·5 % urease dialysed for 5 days, in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 18·8 hours in cc.
5	—	0·26
5	1	4·57
—	1	0·06
Heated to 80° for an hour		
5	—	0·10
5	1	2·59
Heated up to boiling point		
5	—	0·02
5	1	1·68

The conclusion was shown to be correct. Only the yield of ammonia produced by the dialysed urease was smaller if it had been heated. This difference may be ascribed to the total destruction of the co-enzyme which remained fixed. The heating up to boiling point, however, will very likely destroy a certain amount of urease proper.

9. *To what change is the diminution of the activity of urease during preservation to be ascribed?*

Since it has been shown that co-enzyme is in every respect very labile, it is probable that the diminution of the activity of urease during preservation is due to change of co-enzyme.

An experiment was made with urease which was kept with toluene in an incubator at 36°·5 for 15 days. The urease solution was 0·5 % and 5 cc. of it developed 22·6 cc. of N/10 NH_3 in 20·3 hours at the start.

TABLE XXI.

Old urease in cc.	0·5 % fresh urease in cc.	N/10 NH_3 developed in 18·7 hours in cc.
5	—	5·17
5	0·5	10·87
—	0·5	0·11

It is very likely that the co-enzyme was subjected to some change during the preservation, but the data are not sufficiently convincing. Whether the urease proper remained quite intact is also uncertain.

10. *Does blood serum contain co-enzyme beside "auxo-urease"?*

Margarete Falk [1914], and Jacoby and Umeda [1915] studied "auxo-urease" in blood sera which considerably accelerated the urease action. These statements interested me and suggested the attempt to find the co-enzyme of urease in blood.

TABLE XXII.

0·5 % urease dialysed in cc.	Serum of ox in cc.	0·5 % urease heated to 80° for an hour in cc.	0·5 % urease in cc.	N/10 NH_3 developed in 17 hours in cc.
—	—	—	2	1·70
—	1	—	—	−0·02
—	1	—	2	17·17
5	—	—	—	0·26
5	1	—	—	1·21
—	—	5	—	0
—	1	5	—	0·05
—	—	5	1	10·08

As is shown in this table ox serum contains no urease, but a small quantity of the serum accelerates the urease action to an enormous amount; no true co-enzyme was however found in the serum. The urease which was heated at 80° for an hour lost its activity totally, but the addition of the serum could not revive the action at all, while the addition of a small quantity of the fresh urease (co-enzyme) restored the lost activity considerably. The dialysed urease employed was not quite free from the fixed co-enzyme, consequently the addition of the serum caused appreciable increase of activity. This effect was undoubtedly due to an accelerator in the serum, the "auxo-urease."

Discussion.

Since urease loses its activity by dialysis and recovers it by the addition of something contained in fresh solutions, we are justified in calling this something a "co-enzyme," in view of the present ignorance as to what a co-enzyme really is. The facts that it is destroyed by heat and by the action of acid, alkali, etc. suggest a protein nature, but its apparent passage through parchment paper negatives this suggestion, unless it be assumed that it requires for its activity the presence of some diffusible substance. But, again, the fact that addition of the dialysate is ineffective is remarkable and, on the hypothesis suggested, could only be accounted for by supposing that the protein or colloidal activator undergoes some irreversible change. What is distinctly proved is that what is called "urease" can be brought into an inactive state which can be restored to activity by the addition of a trace of some substance or substances which are contained in fresh extracts of soy-bean.

It is clear that further work is necessary to decide which constituents are to be called the enzyme proper and which the "co-enzyme." The possibility cannot be excluded that the residue in the dialyser might contain a substance capable of increasing the activity of fresh urease, although this does not seem probable.

Conclusions.

(1) The urease of soy-bean loses its activity on dialysis. The lost activity is restored by the addition of a small amount of fresh urease. This indicates that the fresh urease contains co-enzyme. The co-enzyme could not be separated, accordingly its nature is not yet known.

(2) It is very likely that the co-enzyme is a system consisting of two groups of components, one of which is dialysable and the other undialysable. The dialysable component undergoes some irreversible change during dialysis.

(3) The co-enzyme consists of two parts, fixed and free.

(4) Heating and dialysis destroy the free co-enzyme first, then the fixed co-enzyme. The last portion of the fixed co-enzyme is found in the precipitate produced by dialysis, resisting the influence of heating and dialysis tenaciously.

(5) The inhibitory effects of heat, acid and alkali are exerted upon the co-enzyme, but not upon the urease proper.

(6) In germination, urease accumulates in the germs of the soy-beans in large proportion, but free co-enzyme is absent.

(7) Although ox serum has an accelerating power, it contains no substance which can be compared with the co-enzyme.

In conclusion I have pleasure in expressing my cordial thanks to Prof. Bayliss for his advice.

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LII. ON THE PREPARATION OF COLLODION MEMBRANES OF DIFFERENTIAL PERMEABILITY.

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(*Received November 25th, 1915.*)

In the course of a study of the active principle of the extract of a parasitic fungus, the writer [Brown, 1915] attempted the fractional dialysis of the extract. For this purpose he first employed membranes prepared by impregnating filter papers with gelatin according to the method of Bechhold [1907]. By varying the strength of the gelatin employed a series of membranes of different permeability was obtained, none of which however was able to hold back completely the active principle of the fungus. Recourse was had, therefore, to collodion membranes. As this technique was new at the time to the writer, extremely discordant results were at first obtained. It soon appeared that with the same collodion solution very great differences in the properties of the resulting membranes could be produced by variations in the mode of treatment. A revision of this technique was undertaken, involving on the one hand a critical examination of the methods hitherto described and on the other the elaboration of methods which, to the present knowledge of the writer, constitute a new departure in the preparation of collodion dialysers.

It is not proposed to enter fully into the somewhat scattered literature relative to collodion membranes. A fairly complete account of the older literature on the subject is given in a paper by Bigelow and Gemberling [1907].

In practice collodion membranes are employed in the form either of flat sheets or of thimbles. Each type has certain special advantages. Thus the former lends itself more readily to osmometric work inasmuch as it can

easily be backed by some arrangement to take up the pressures developed; for purposes of dialysis the thimble form is simpler in manipulation as it does not require to be tied to any glass structure.

Collodion thimbles may be formed in two ways. A test tube is dipped into a collodion solution, and the resulting film left to undergo a certain amount of evaporation of the solvent. The film is then hardened by immersion in water, and the membrane so produced is stripped off by eversion. This is the method used by Malfitano [1904], by Duclaux [1907], etc. By other experimenters the membranes are formed inside the tube. The latter is filled with collodion solution which is then poured out, and the film of collodion remaining is allowed to drain for some time. The film is hardened by addition of water, and the resulting membrane readily shrinks away from the glass tube. This is the method described by Smith [1905], Bigelow and Gemberling [1907], Lillie [1907], Walpole [1915], etc.

Sheet collodion is prepared by pouring thin collodion solution over a glass surface which has been carefully levelled [Walpole, 1915] or on a mercury surface [Bigelow and Gemberling, 1907]. It is hardened in the usual way.

Numerous attempts have been made to prepare collodion membranes of uniformly varied permeability. These now call for detailed mention.

Bechhold [1907] prepared collodion films by impregnating filter papers with solutions of different strengths of collodion in glacial acetic acid. Impregnation was carried out *in vacuo*, and the filter papers after draining for a short time were hardened by immersion in water. The permeability of the membranes obtained was found to vary inversely with the strength of the collodion solution used.

Bigelow and Gemberling [1907] used a solution consisting of 3 g. commercial pyroxylin in 75 cc. ether + 25 cc. alcohol. They varied the permeability by varying the time allowed for evaporation of the solvents. No means of standardising this process were described.

Malfitano [1910] observed that the permeability of collodion thimbles diminished as they were allowed to dry in air; also that it could be diminished by heating the membrane in water. With similar treatment in other respects, a collodion richer in alcohol gave a more permeable membrane than one rich in ether. No details of a systematic nature were given.

Schoep [1911] describes a method which does not seem to have received the recognition that it deserves. He prepared membranes from pyroxylin-

castor oil solutions in alcohol-ether. The following represents the type of formula used by him:

Pyroxylin	4 g.	
Castor oil	3·5 g.	
Alcohol and ether	92·5 cc. (alcohol and ether in ratio 1 : 8).	

In order to vary the permeability of his membranes, Schoep added glycerol in various quantities to the pyroxylin-castor oil solution. The membranes were then formed inside a glass tube and allowed to drain and dry to constancy. On testing these membranes he found that their permeability increased with increasing glycerol concentration used.

Walpole [1915] relied mainly upon sheet collodion prepared by pouring a 3·5 times diluted stock solution of Schering's celloidin [13·45 per cent. by weight in equal parts of absolute alcohol and ether—thus approximately a 4 % solution] over a horizontal glass plate. The amount of solution used and the area of the resultant membrane being known, the amount of nitro-cotton per sq. cm. could be determined. This quantity (m) is used in describing the membrane. In order to obtain membranes of varied permeability Walpole varied the drying period. To determine how far drying had proceeded, he took sample portions of the film, washed them in water, and determined their thickness by means of a micrometer. With the same film, the thickness of the resulting membrane decreased as evaporation proceeded, so that by determining the former the progress of evaporation could be followed. Walpole found that the membranes so obtained could be described fully, for purposes of reference and reproduction and as a guide to their properties, by stating the kind of nitro-cotton used, the weight of it per sq. cm. they contained, and the "wetness," i.e. the ratio of the weight of a piece of film to its weight when dried to constancy.

The method of varying the permeability discovered by the writer is as follows. Collodion membranes are prepared and then completely air-dried whereby they become highly impermeable; they are then placed in solutions of alcohol in water for a suitable time. When subsequently washed in water these membranes are found to show a permeability which decreases regularly with the strength of the alcohol employed.

Thus while in the methods of manufacture hitherto employed, successful grading was a matter of skill which was not always definable, the present method is merely mechanical and the only skill required is in the making of the initial uniform air-dried membranes.

Standard method of preparation of air-dried membranes.

An 8 % solution of Schering's celloidin in an equal mixture (by volume) of absolute ethyl alcohol and ether is used. The membranes are formed on the outside of a test tube (1 cm. diam.). The latter is dipped in the collodion solution to a depth of 8–10 cm., and after a few seconds' draining is placed in a vertical position inside an inverted conical flask ($\frac{1}{2}$ litre capacity), being held by an ordinary cork which fits the neck of the flask. The purpose of the flask was in the first instance to limit the evaporation of the solvents so as to ensure an even film of collodion. By manipulation of the tube after insertion into the flask the length of the membrane can be increased. Drainage is allowed to proceed for 5 minutes after which the tube is withdrawn and *immediately* immersed in water. After a time (about 1 minute) the membrane is stripped off by eversion. For this purpose use is made of the "runnels" of collodion, which have by now become solidified. These on being cut across serve as handles in pulling the membrane off. The membranes are then trimmed, washed for a short time in water, and laid out to dry at laboratory temperature. Drying takes place with shrinkage and is completed overnight. The membranes are now ready for the alcohol treatment.

The shrinkage consequent on drying is a factor of very considerable importance in the present technique. A membrane which dries with excessive shrinkage will show at the finish a diminished surface over which diffusion may take place, along with increased thickness, and will not afford results comparable with those of a less shrunken membrane. Great care has therefore been exercised in examining the factors determining this shrinkage and in devising means for its proper control. What these factors are will appear from the following.

Characters of a normal membrane previous to drying off.

The characters which were examined in this connection were:

(1) permeability;

(2) water-content;

(3) thickness, before and after drying; shrinkage on drying, both longitudinal and circumferential.

A normal membrane is permeable to all crystalloids and allows slow diffusion of dextrin. It is more permeable at the closed end and may be relatively impermeable towards the open end. This is shown by the fact

that with certain dyes the inner surface of the membrane stains more rapidly in the neighbourhood of the closed end than towards the opening. Again the permeability of the outer surface is greater than that of the inner surface.

After washing in water, these membranes contain from 39·5 to 43·0 % of water (in terms of total weight).

It is obvious from the foregoing that with membranes of the present type the wetness gives no certain indication of the permeability as the membrane is not uniform on both sides.

The following method was adopted in measuring the shrinkage relationships of these membranes. The membrane was split from top to bottom along one side; the circumference was determined at top (open) and bottom (closed) ends, and also the thickness (by means of a micrometer) across the top, middle and bottom. In order to determine the degree of longitudinal shrinkage, a distance of 4 cm. was measured and marked by cuts in the long direction of the membrane. A repetition of these measurements in the case of the air-dried membrane gave data by which the shrinkage relationships could be followed.

The following series of measurements is typical of the present type of membrane (viz. a membrane which was drained 5 minutes in the flask and was immediately immersed in water). In the micrometer readings, each unit is equivalent to $5\,\mu$.

Membrane before drying.

Circumference = 4·53 cm. at top; 4·50 at base.

Micrometer readings.

5·2, 5·8, 5·4, 5·5, 5·3, 5·0, 5·1 near top.	Av. = 5·33.
5·7, 5·7, 5·6, 5·6, 5·8, 6·0, 5·9 near middle	Av. = 5·76.
6·3, 6·3, 6·6, 6·3, 6·2, 6·4, 6·2 near base.	Av. = 6·33.

After drying.

Circumference = 4·15 cm. at top; 4·05 at base.
Marks 4 cm. apart have shrunk to distance 3·63 cm.

Micrometer readings.

4·0, 4·0, 4·0, 4·0, 4·0, 3·9, 3·7 across top.	Av. = 3·95.
3·6, 3·7, 3·5, 3·6, 3·5, 3·5, 3·4 across middle.	Av. = 3·55.
3·3, 3·3, 3·2, 3·2, 3·2, 3·2, 3·2 across base.	Av. = 3·23.

In connection with this series of figures, the following points are noteworthy:

(1) At the same level of the thimble there is a variation in thickness, both in wet and in air-dried membranes. This variation is greatest near the open end. This can be ascribed to unevenness of drainage. It is better to discard the top part of each membrane.

(2) There is a variation in thickness from base to top of the same membrane.

In the case of air-dried membranes, thickness is found to increase from closed to open end. From a large number of observations, this rule has been found to be general. No such rule is however applicable to the corresponding undried membranes. Thus in the preceding case, the undried membrane was thicker towards the closed end, but nevertheless the closed end became the thinnest part of the membrane when the latter was dried off. Correlated with this we find the basal region to be the most permeable part of the wet membrane. In many cases however the undried membranes are found to show the same increase in thickness from the closed end upwards. The cause of these variations will be discussed below.

(3) The shrinkage relationships may be summarised thus:

Length before drying	=100	Length after drying	=90·75
Circumference before drying	at top =100 at base =100	Circumference after drying	at top =91·6 at base =90·0
Thickness before drying	at top =100 at base =100	Thickness after drying	at top =74·1 at base =51

Measurements of the above type have been carried out for a large number of membranes. The following general rules can be drawn:

(*a*) Longitudinal and circumferential shrinkage are approximately the same. Shrinkage is slightly greater at the closed end. Shrinkage in different membranes of the same type is remarkably uniform; thus of more than 30 measurements made, all lay within the limits 87 and 92, the majority lying between 88 and 90.

(*b*) Shrinkage in thickness is more variable. The thickness of the dried membrane varies from 50 to 75 % of the undried. Shrinkage in thickness is always greater than superficial shrinkage. This anisotropy in respect of shrinkage is invariable with these membranes.

The results so far obtained may be conveniently summarised at this stage.

By following the method described on p. 594, membranes can be obtained which on being air-dried present the following features: thickness $= 20 \pm 5\,\mu$; tendency to greater thickness towards the open end; longitudinal and circumferential shrinkage very constantly 10 %.

Before drying, these membranes show a different permeability in their different parts; in this state therefore they are of no use whatever in cases where anything like exactness is required in dialysis work.

To attain a high degree of uniformity of the air-dried membranes certain precautions must be taken. The most important of these will appear from the following discussion of the effect of varying the conditions on the nature of the resulting membrane.

(i) *Effect of allowing free evaporation of the solvents.*

In the method of preparation described above, the membranes after draining in the flask are immediately plunged into water. The effect produced by even a short sojourn in the free atmosphere of the laboratory is very remarkable. This will appear from the following data:

The membrane is drained for 5 minutes in flask; on taking out it is held for 1 minute in air. Such a membrane will be described by the notation (5, 1); by the notation (5, 0) is meant a membrane which after 5 minutes' draining in the flask is immediately plunged into water.

Measurements of a (5, 1) *membrane.*

Circumference at top and bottom = 4·55 cm.
Micrometer readings 17·0, 17·0, 19·0, 18·7 across top. Av. = 17·9.
17·5, 16·3, 17·5, 18·9 across middle. Av. = 17·55.
15·3, 15·0, 15·9, 15·1 across base. Av. = 15·3.

After drying.

Circumference = 3·2 cm. at top; 3·1 at base.
Distance between 4 cm. marks = 2·8 cm.
Micrometer readings 8·0, 7·0, 6·5, 6·5 across top. Av. = 7·0.
7·0, 7·0, 6·3, 6·8 across middle. Av. = 6·8.
5·7, 6·1, 6·1, 6·1 across bottom. Av. = 6·0.

Thus the shrinkage relationships are summarised in the following table.

Length before drying		= 100	Length after drying		= 70·0
Circumference before drying	at top	= 100	Circumference after drying	at top	= 70·3
	at bot.	= 100		at bot.	= 68·1
Thickness before drying	at top	= 100	Thickness after drying	at top	= 39
	at bot.	= 100		at bot.	= 39

There is thus in this case excessive shrinkage on drying. Membranes of this type are before drying highly permeable—much more so than membranes of the type (5, 0). They are slowly permeable to aniline blue, strongly permeable to dextrin and allow crystalloids to pass with great rapidity. Correlated with this they show a high water-content; thus weight of dry membrane = 100; weight of wet membrane = 170.

The above figures are typical of all membranes of this type. The longitudinal and circumferential shrinkage borders very constantly on 30 %; the shrinkage in thickness approximates closely to 60 %.

A comparison of the measurements of a (5, 0) and a (5, 1) membrane is instructive. The unit of thickness is, as before, $5\,\mu$.

(5, 0). Before drying. Thickness = 5–7.
After ,, ,, = 3–4.
Longitudinal and circumferential shrinkage = 10 %.
Shrinkage in thickness = 25–50 %.

(5, 1). Before drying. Thickness = 15–18.
After ,, ,, = 6–7.
Longitudinal and circumferential shrinkage = 30 %.
Shrinkage in thickness = 60 %.

It will be noticed that while shrinkage in thickness is greater in membranes of the second type, the final thickness of the latter when dried is about twice that of the former.

It will thus be seen that unequal evaporation of the solvents produces unequal shrinkage in the resultant membranes. The fuller discussion of these results will be postponed until the variation of a second factor has been examined.

(ii) *Effect of varying the time of drainage.*

The time of drainage within the flask was varied from 1 minute up to 10 minutes, and the resulting membranes tested as to their permeability, thickness in the wet condition, and shrinkage on drying. The following table gives the figures obtained. The notation in the case of the membranes is that already described (p. 597).

Membrane	Thickness when wet	Longitudinal and circumferential shrinkage	Thickness when dry
(1 0)	6–8	5–6 %	4·4–6·3
(1 ½)	17–19	20 %	5·7–6·9
(1 1)	19–20·4	25–27·5 %	6·7–8·0
(3 0)	6·3–7·9	7·5–10 %	3·7–4·8
(3 1)	17·0–20·8	28–30 %	6·2–7·2
(5 0)	5·3–7·1	8–12 %	3·3–4·5
(5 ¼)	14	22·5 %	5·1–5·7
(5 ½)	13·5–18	20–25 %	5·4–7·8
(5 1)	16·7–20·4	30–32 %	6·0–7·4
(5 2)	20·8	30 %	7·8
(10 0)	5·0–5·25	11–13 %	3·0–3·1
(10 1)	12·6–13·6	30 %	5·1–5·4

From the above table it is clear that by prolonging the period of drainage a slow increase of the shrinkage obtained on drying the resultant membrane takes place; that when once the membrane is taken out and allowed to

evaporate freely, a rapid change sets in, and the shrinkage soon reaches as much as 30 %. In this technique therefore it is imperative that the membranes be taken from the flask and immediately immersed in water. The rapid execution of this process involves a certain amount of dexterity only attainable by practice. The writer has convinced himself that it is quite possible to reduce the shrinkage variation to less than 5 %. Should it be considered necessary to bring this factor under better control, it is obvious that an arrangement could be set up whereby the films could be hardened without being exposed at any time to rapid evaporation of the solvents in the open air.

By lengthening the time of drainage the thickness of the resultant membrane diminishes, but only slowly. Even after 10 minutes' draining, the membranes when dried are thicker at the open end than near the closed end. Uniformity in thickness is a condition which is approximated to only after very prolonged drainage.

The membranes which have been allowed to evaporate freely for some time all show high permeability. In the case of membranes which are immediately transferred to water, permeability increases with time of drainage. Thus a (1, 0) membrane is impermeable, except at the closed end, to neutral red; a (10, 0) membrane shows a fairly uniform medium permeability throughout. With intermediate drainage, the membranes show a gradation from a more permeable region near the closed end to a less permeable region near the open end.

A membrane of the (1, 0) type when placed in water becomes strongly milky in appearance except just at the closed end which is clear. By longer drainage, the milkiness is reduced, and in the case of a (5, 0) membrane becomes very slight. Membranes of the strongly shrinking type are always glass-clear.

It is convenient at this point to explain the principles underlying the phenomena above recorded. For this purpose it is necessary to anticipate somewhat.

Membranes prepared from a solution of collodion in ether alone are highly impermeable, are white, have a low water-content, and show a very small shrinkage on drying. This statement applies all the more markedly when practically no ether is allowed to evaporate from the film before it is placed in water. Membranes made from a solution of collodion in alcohol alone are, on the other hand (unless a large proportion of the alcohol be allowed to evaporate), highly permeable, are clear, have a high water-content

and undergo strong shrinkage on drying. The various types of membrane obtained above represent intermediate stages of this series.

Now when a film of collodion on a test tube is exposed to free evaporation, the first result is the rapid diminution of the ether content. The membrane therefore lies near the alcohol end of the series, and a highly permeable, strongly shrinking membrane is obtained. Membranes of the type (1, 1), (3, 1), (5, $\frac{1}{2}$), (5, 1), (10, 1) are nearer to the alcohol end of the series, those of type (1, 0), (3, 0), (5, 0), (10, 0) are nearer to the ether end. That the (1, 0) membrane approximates more closely to an ether-collodion membrane than does the (10, 0) is due to the fact that the atmosphere inside the flask requires some time to reach equilibrium, and also to the fact that with the apparatus used a slow leakage of ether took place at the neck of the flask. That the basal (closed) end approximates more closely to the alcohol type of membrane than does the other is to be set down to the fact that in the earlier stages of drainage when evaporation is most rapid the closed end of the film (which is uppermost) has reached its limiting thinness, whereas the lower portion is protected by the excess collodion which is flowing past. This inequality, as has been already stated, is greatly diminished after about 5 minutes' drainage.

It will conduce to clearness at this stage if the essential features of the technique described above be emphasised. This is most conveniently done by stating the reasons which lead the writer to recommend each particular detail of the method described on p. 594.

It should be stated first of all that membranes of the strongly shrinking type are to be avoided for reasons of efficiency in dialysis. With equal dimensions at the start, the capacity of a membrane which has shrunk by 30 % bears a ratio $7^3 : 9^3 = 1 : 2{\cdot}1$ to that of one which has shrunk by 10 %—i.e. it is less than half. The thickness of the much shrunk membrane is also greater, so that with the same intrinsic permeability, diffusion is slowed down. It need hardly be added that in setting up any series of graded dialysers, membranes showing different degrees of shrinkage must be avoided.

Use of the conical flask. When a test tube is dipped into an 8 % collodion solution and withdrawn, a drop of collodion forms at the closed end of the tube. When the tube is placed vertically with closed end upwards, this drop must be made to travel the whole length of the film, otherwise extreme irregularity in thickness of the membranes results. When the films are allowed to evaporate freely there is a tendency for the excess collodion to stick half way, and very uneven drainage results. This tendency is

especially marked in warm weather. Again, even if drainage is fairly uniform, the membranes are found on drying to be of the strongly shrinking type. The use of the flask is to obtain even drainage along with a minimum of shrinkage on drying.

Strength of collodion used. With increase in concentration above 8 %, a tendency to uneven drainage sets in. Thus 10 % collodion after 5 minutes' drainage gives distinctly less uniform membranes than does an 8 % solution. With a 5 % solution, even drainage is soon secured. The membranes are however more liable to tear on stripping from the tube, and are found to shrink strongly. The latter feature is due to the fact that on account of the comparative fluidity of a 5 % solution, only a small amount of solution is carried into the flask on the tube, and consequently a considerable percentage of the solvent (especially ether) is evaporated before the atmosphere of the flask becomes saturated.

Time allowed for drainage. For the membranes normally employed, a period of 5 minutes' drainage was allowed.

For the first 1–2 minutes, drainage is still proceeding rapidly. From 3 minutes onwards only a very slow drainage takes place. Membranes which have drained 3 minutes show greater variation between closed and open ends than do membranes of the normal type. They also show a much greater tendency to milkiness. The latter objectionable feature is reduced to very small dimensions in the case of membranes which have drained 5 minutes. The only objection to drainage longer than 5 minutes is the time thereby taken up.

In carrying out the routine of preparing membranes, the experimenter cannot afford to abate any consideration which tends to uniformity. Each process in the manipulation should be standardised. In particular, the membranes should be made at a standard temperature (not higher than 20° C.), and as nearly as possible the same amount of solution should be taken up by the test tube in each case.

Summary. By carrying out the above routine, membranes can be obtained which show a shrinkage on drying of 10 ± 2 %. The dried membranes vary in thickness from 15 μ at the closed end to 20–25 μ at the open end; at any given level in the same membrane a variation in thickness of 10 % is liable to occur.

Treatment with alcohol.

The stock alcohol (ethyl) employed was of specific gravity 0·7956 (at 16° C.), corresponding thus to 99·36 % absolute (by weight).

By an x % alcohol in the following account is meant an alcohol-water mixture containing x cc. of stock spirit + $(100 - x)$ cc. water.

The air-dried membranes are soaked in the alcohol mixtures for 24 hours at 20° C.; they are then transferred to distilled water and thoroughly washed over a period of 1 day. They are then ready for use.

When uniform air-dried membranes are used, the permeability is determined by the concentration of alcohol employed. It may thus be fully specified in terms of the alcohol percentage. By the phrase "a 75 % membrane" is meant an air-dried membrane which has been soaked in 75 % alcohol for 24 hours at 20° C., and subsequently washed in water.

The series of membranes treated with increasing percentages of alcohol is thus a series showing gradually increasing permeability. The limits of the series are on the one hand 0 %, on the other about 97 %, above which point the membrane goes into solution. The most permeable membrane that has been systematically used is a 96 % one; 97 % membranes have also been prepared but are extremely fragile and apt to rupture. Membranes more permeable still have been prepared by allowing a shorter imbibition period in 98 % alcohol. It is possible therefore to extend the series, thereby obtaining extremely permeable membranes, but they are of doubtful value unless means be taken to support them and protect them from mechanical injury.

In the following work these membranes were set up in specimen tubes of 1·7–2 cm. diameter. In cases where estimations of diffusion were made, tubes of 1·8 cm. diameter were alone used. The membranes were filled with the solution to be tested to a distance of about 1 cm. from the top, and the space between the membrane and the tube filled to the same level with water.

The following substances were used:

NaCl(m), NH_4Cl(m), Na_2SO_4(m), $CuSO_4$ (sat.), $K_4FeC_6N_6$ (sat.), $K_2C_2O_4$(m), glucose (m and m/5), picric acid (sat.), $KMnO_4$ (0·5 %).

Bismarck brown, methylene blue, eosin, neutral red, safranine, aniline blue, Congo red, night blue (all 0·1 %).

Dextrin (0·5 %), starch (0·1 %), litmus (Kahlbaum).

All the solutions were made up in water.

In order to prove that a continuous gradation in permeability is obtained by the alcohol treatment, four main methods were employed.

I. Using a suitable crystalloid and membranes within a given range, the amount of endosmosis of water in a given time increases with the strength of alcohol used.

This method is capable of great elaboration, viz. by constituting the membrane an osmotic cell with capillary tube attached, and measuring the rate of endosmosis of water. This method of testing the variation of permeability with alcohol treatment, and the degree of uniformity of membranes treated in the same way, has not been carried out in detail as yet and only a few rough measurements of the alteration of level between the liquid inside the membrane and the water outside are at present available. In most cases no measurement was taken, but a note made to the effect that rate of endosmosis increased with strength of alcohol used. These measurements were made incidentally to determining the permeability of these membranes to various salts.

The following figures illustrate the type of result observed:

0 %, 60 %, and 70 % membranes set up with molar potassium oxalate.

After 24 hours, endosmosis of water in 0 % = 0·2 cm.
" " 60 % = 3·5 "
" " 70 % = 4·6 "

The increased endosmosis in 70 % takes place in spite of a slight passage of the oxalate through the membrane. In fact, increased endosmosis of water with strength of alcohol used takes place long after the membrane has become quite permeable to the osmotic substance. In such a case, of course, the endosmotic effect will prove to be transitory.

II. Within certain limits, as regards nature of membrane and nature of dye, the amount of staining of the membrane in a given time increases with the strength of alcohol used.

This method has proved of great value, especially for revealing what amount of irregularity in permeability may exist in different parts of the same membrane. The present test is certainly the most accurate from this point of view that is known to the writer. Though much use has been made of this test, only the cruder outlines are as yet worked out. The method lends itself readily to exact colorimetric work.

The following account of a particular experiment will illustrate how the method works out:

50 %, 60 %, 70 %, 80 % membranes set up with neutral red solution inside, water outside.

After 1 hour's staining, all membranes are thoroughly washed in water.

A continuous gradation in staining effect is seen, the latter being least in the 50 % and greatest in the 80 % membrane. The membranes are again set up with neutral red and left for 24 hours. After this time, the dye has passed through the 80 % membranes. On cutting all the membranes open, the difference in staining between the 70 and 80 % membranes is now found to be slight; a marked difference is still shown between 50 and 70 %. Even after prolonged staining, this difference is not effaced—that is, e.g. the intensity of staining of a 60 % membrane never reaches that of a 70 % one.

From an extended series of experiments of this sort, a continuous gradation in general staining effect with varying strength of alcohol has been established. This is readily done in the case of 0 %, 30 %, 40 %, 50 %, 60 % and 70 % membranes with neutral red solution; by 24 hours' staining with aniline blue, a similar gradation can be established in the case of 70, 75, 80, 85 and 90 % membranes. Staining of 80, 85, and 90 % membranes with neutral red takes place too rapidly to allow of a satisfactory differentiation.

In applying this method to the detection of irregularities in permeability of different parts of the same membrane, the latter should be stained with a dye which is unable to pass through it. Thus neutral red was employed in the case of 30–70 % membranes; aniline blue for 70–90 % membranes. In cases where the dye is able to pass through the membrane, the staining rapidly becomes so intense that variations are no longer noticeable.

The following are the results obtained:

(1) With 70 % and higher membranes, staining is completely uniform, apart from a limited number of small spots at which the membrane is less permeable.

(2) With membranes less than 70 % there is, in addition to the above irregularity, a tendency to uneven staining, the membrane being more permeable near the closed than near the open end.

(3) When the outside of the membrane is tested in this way, greater irregularity may occur. This irregularity is correlated with the presence of milkiness, the milky patches staining more strongly. This effect is shown equally well in the air-dried (0 %) membranes, and with such a dye as aniline blue. On cutting such membranes and examining microscopically, the staining effect is seen to stop in the middle of the membrane.

With glass-clear membranes the degree of staining of the outer surface is uniform and equal to that of the inner surface.

It may be remarked here that the presence of a number of small less permeable spots has been observed in the case of membranes of all kinds, no matter how made and no matter what the duration of the alcohol treatment. Their cause is unknown. From their small size, their effect on the general permeability of the membrane is negligible.

III. The amount of a particular substance passing through in a given time increases with strength of alcohol used.

Increased permeability with varying strength of alcohol has been followed over a range of 30 % to 96 % by measuring the rate of diffusion through the various membranes of certain substances. The membranes were set up as usual, the containing specimen tubes being chosen of equal diameter. The diffusing substance was placed inside, and the external space filled to the same level with water. The substances employed were such that no appreciable disturbance of levels (and volumes) was produced by endosmosis of water. Diffusion was carried on in a thermostat at 20° C.

(*a*) Diffusion of potassium permanganate (0·5 % solution). The amount diffused through in 24 hours was determined by titration with a 0·1 % $FeSO_4$ solution in dilute H_2SO_4.

Membranes—a double series of 30, 40, 50, 60, 70 %.

In all cases the tubes were filled to the same height, and at the end of the diffusion period the amount of solution inside the tube and outside the membrane approximated closely to 5 cc.

		Membrane				
		30 %	40 %	50 %	60 %	70 %
cc. of 0·1 % $FeSO_4$	Series *A*	0	1·6	10·6	31·2	76·5
	Series *B*	trace (< 0·10)	1·55	8·8	32·5	70·2

The difference between 60 % and 70 % is much greater than the figures represent. At the time of examining, equilibrium had nearly been reached between the outside and inside solutions.

The grading can also be seen by noticing how long a time elapses from filling the membranes till the first traces of colour appear in the liquid outside.

Thus in the case of the foregoing membranes, this happened within 1 minute in both cases with 70 %; in 4–5 min. in both with 60 %; in 25 min. in both with 50 %; in about 2 hours in both with 40 %; while in the case of 30 % membranes, nothing to a trace appears in 24 hours.

On washing these membranes staining is seen to have taken place; on

again air-drying them they are found to be impermeable to permanganate. The presence of holes is thereby excluded. It is probable that the membranes are slowly attacked—at least the permanganate becomes turbid when kept in a membrane for about a week.

(*b*) Over the range 70 to 95 %, the grading can be followed by using methylene blue. Over the range 70–80 % this has been done in considerable detail (see Uniformity tests below). Over the wider range grading is shown by the time required for the dye to appear in the outside liquid. The following figures illustrate this point:

70 %	75 %	80 %	85 %	90 %	95 %
about 6 days	1 day	8–12 hours	4 hours	70 min.	12 min.

(*c*) Over the range 85 to 94 %, with variations of 1 % in alcohol treatment a continuous gradation of permeability can be shown by using dextrin as diffusing substance.

A dextrin solution of 0·5 % was used and dextrin was tested for in the external solution by adding 1 drop of N/10 iodine. After 24 hours, no trace appears in 85 %; a trace in 87 %, and this becomes stronger in each successive member of the series. The gradation becomes more rapid towards the upper end of the series.

(*d*) Over the range 92–96 %, the variation of permeability may be shown by use of neutral litmus.

After 10 days' dialysis, the following table gives the strength of dye in the external solutions:

92 %	93 %	94 %	95 %	96 %
0	trace	0·08	0·13	0·33

the internal solutions being of strength 1.

IV. The space relationships of the membrane (amount of water, swelling in all three dimensions) vary continuously with strength of alcohol used.

Gradation is shown over the whole range by measurement of the *water-content*. In making these determinations, the closed end of the membranes was cut away, and the cylindrical portion left was cut open. In determining the wet weights, both surfaces of the membrane were dried rapidly between filter paper; weighing was carried out in a closed bottle.

The gradation is shown in the following table. In each case the weight is given in terms of an original air-dry weight of 100. (Air-dried membranes contain about 3 % water.)

Membrane %	Wet weight after washing	Weight on again drying
0	106·0	100·0
50	111·0	99·3
70	115·6	98·6
80	122·4	98·2
90	141·9	97·0
92	160·5	93·8
94	208·5	89·1
96	387·8	74·8

A parallel series of measurements of the strongly shrinking (5, 1) membranes gave essentially similar results, except that the water percentage in the case of these tended to be higher near the upper end of the series than that of the preceding type.

It will be noticed that the increase in water-content is gradual at first and becomes extremely rapid above 94 %; also that the membrane loses in dry weight with the treatment; that this loss is slight up to 90 %, but becomes very considerable at 96 %.

In the same way the length, circumference and thickness of the air-dried membranes increase continuously when the latter are immersed in alcohol solutions of increasing strength. The following tables show these effects.

(*a*) *Membranes which show* 10 % *shrinkage on drying.*

The measurements of L (length) and T (thickness) are given on the basis of a length and thickness in the air-dried condition of 100. Longitudinal and circumferential extension have never been found to differ appreciably.

Membrane %	In alcohol before washing		After washing		After drying	
	L	T[1]	L	T	L	T
0	101·7	—	101·7	100·0	100·0	100·0
50	103·7	—	102·0	108·3	97·8	102·8
70	105·8	—	102·0	110·5	—	105·2
80	108·6	—	103·3	118·9	96·7	105·4
90	112·9	—	107·0	162·2	94·7	108·1
92	115·8	—	107·7	200·0	92·1	114·3
94	119·7	—	111·7	280·0	89·5	116·7
96	132·1	—	120·9	362·0	76·7	*

[1] Readings not taken on account of softness of membranes, especially in the higher percentages of alcohol.

* Becomes very crinkled; micrometer reading valueless.

The features brought out by Tables (*a*) and (*β*) apart from the continuous gradation are:

(1) Swelling is anisotropic. Thus in 94 % alcohol, the longitudinal extension is 12 %; the extension in thickness is 180 %. This applies to both sets of membranes.

(β) Membranes which show 30 % shrinkage on drying.

Membrane %	In alcohol before washing		After washing		After drying	
	L	*T*[1]	*L*	*T*	*L*	*T*
0	102·0	—	101·0	103·5	100·0	100·0
50	104·4	—	[100·5]	107·0	[96·8]	*
70	107·4	—	102·3	124·5	96·7	*
80	108·3	—	102·5	129·6	95·5	*
90	119·7	—	110·0	150·0	94·1	111·0
92	126·8	—	118·3	186·0	—	112·0
94	138·4	—	125·6	300·0	87·2	117·5
96	157·3	—	139·1	410·0	65·1	*

[1] Readings not taken on account of softness of membranes, especially in the higher percentages of alcohol.

* Impossible to obtain a reliable measurement on account of crinkling on drying.

(2) A normal (10 % shrinking) membrane is restored to its original length and circumference by immersion in alcohol of about 94 %. The thickness and, from table on p. 607, the water-content are much increased. Correlated with this is increased permeability of a 94 % membrane as compared with that of the membrane as originally prepared.

(3) With membranes of both types, longitudinal extension is negligible in membranes of 80 % and under; in these the effect of the alcohol treatment shows itself mainly in increase in thickness.

(4) With the higher percentages of alcohol, the strongly shrinking membranes show a higher extension than do the corresponding members of the normal series. Nevertheless the original dimensions are only reached at a higher alcohol percentage, viz. 94–96 %.

(5) The higher the percentage of alcohol employed, the greater is the subsequent shrinkage in length on drying (as shown by comparison of 2nd and 3rd *L* columns in both tables). This increased longitudinal shrinkage is compensated in part by increase in thickness on drying.

While the size relationships above described afford a very complete proof of the continuity in the series of membranes produced by alcohol treatment, their importance must not be over-emphasised. Collodion is a plastic substance, and therefore expansion takes place in the direction which is easiest. The anisotropy above observed is to be explained in this way. It does not at all mean that any kind of structure is to be postulated with regard to the membrane. The higher coefficient of longitudinal expansion of thick than of thin membranes is also to be explained in the same way—the thicker the membrane the more does it tend to expand isotropically, and similarly the thinner the membrane the greater is the tendency for expansion to be

anisotropic. As an example of the latter may be cited some experiments with very thin membranes prepared in a way to be described later in which a longitudinal extension of 3–4 % was accompanied by an increase in thickness of 200 %.

In accordance with this principle, the permeable membranes obtained after alcohol treatment are more uniform in thickness than the dried membranes from which they were derived, and the tendency to uniformity is greater the higher the alcohol percentage.

Uniformity tests.

The diffusion experiments with potassium permanganate tabulated on p. 605 indicate the type of agreement obtaining between the corresponding members of two similarly treated series. More detailed tests of the same type were carried out within the range 70–80 % with methylene blue.

In one experiment, 15 membranes prepared in succession on the same day (membranes not selected in any way and of ordinary uniformity) were treated, 3 in 70 % alcohol, 3 in 72·5 %, and so on with 2·5 % intervals to 80 %. These were set up with methylene blue solution, and the rate of diffusion estimated from day to day by comparing the external liquids with a dilution series of the original methylene blue solution. The members of this series were of strength—1, 2, 4, 8, 16, 32, 64, 128, 256, the last being of strength equal to one-fourth that of the original methylene blue solution. The figures in the following table give the members of this series to which the various dialysates corresponded.

Membrane	1 day	2 days	3 days	4 days	5 days	6 days	7 days
70 (1)	0	0	0	0	0?	2—1	2
70 (2)	0	0	0	0	0	0?	trace
70 (3)	0	0	0	0	0	0?	trace
72·5 (1)	0	2—1	8—4	8	16	32—16	32—
72·5 (2)	0	2—1	4	8—4	8+	16—	16
72·5 (3)	0	2—1	4	8—4	8+	16—	16
75 (1)	trace	16—	32—	32+	64—	64	64+
75 (2)	trace	8	16+	32	64—32	64—	64
75 (3)	trace	8—	16	32—	64—32	64—32	64—
77·5 (1)	8—	32	64—	64	128—64	128—	128—
77·5 (2)	8—4	32	64—	64—	64+	128—64	128—
77·5 (3)	8	64—32	64	64+	128—	128—	128—
80 (1)	16+	64—	64+	64+	128—	128—	128—
80 (2)	16	64—	64+	64+	128—	128—	128—
80 (3)	32—16	64+	128—64	128	128	128	128

After 4–5 days no further dialysis takes place in the 80 % membranes, so that the less permeable 77·5 % membranes overtake them. On examining the liquid inside the 80 % dialysers on the 5th day, it was found that its

strength was in each case about one-eighth that of the original solution. This loss in strength is due to the strong adsorption of dye by the membrane. The adsorptive capacity of these membranes increases with permeability. The amount of dye therefore which passes through the more permeable membranes in the above table is less than would be the case if the internal solutions were kept up to full strength.

A second test on a similarly large scale was carried out, 6 membranes of 75 %, 6 of 77·5 %, and 6 of 80 % being used. The results obtained were essentially the same as in the above case.

Throughout these experiments special attention was directed to the comparison of the most permeable membrane of a given alcohol number with the least permeable of the next higher number. On the basis of these observations, it can be stated that the most permeable membrane of a certain number was in all cases less permeable than the least permeable of those of number 2·5 % higher. Thus whatever be the variations among the individual membranes, these are not sufficient to mask the effects of the differential alcohol treatment—in other words in the region 70–80 % an alcohol treatment which varies by 2·5 % steps definitely produces continuous gradation in an average set of membranes. At the same time however the maximum variation among similarly treated members may be greater than the least gap which obtains between members of two lots the treatment of which differs by 2·5 %. Thus it is probable that with unselected membranes, 2·5 % more or less represents in the region 70–80 % the smallest variation of alcohol treatment which can be reckoned on to mask individual variations and to produce in all cases continuous gradation.

It is reasonable to ascribe the variations here recorded to variations in the original air-dried membranes (especially individual variations in thickness), and therefore it may be expected that with improvement in the method of manufacture a finer gradation will be rendered possible.

At higher points in the series a finer gradation in terms of alcohol percentage is possible. Thus from about 85 % upwards, continuous grading can be obtained by a series of alcohols varying by 1 %. In the region 90–96 %, especially towards the upper end, smaller differences in alcohol treatment will suffice to produce continuous grading. In the region 96–98 % imbibition increases so rapidly with alcohol percentage that a large range of membranes is included within this narrow limit. The accuracy of the grading in this region therefore would probably be found to be limited by the accuracy obtainable in preparing the alcohol mixtures.

From 40–70 % a variation of 10 % in alcohol treatment produces continuous gradation. The air-dried membranes show differences in permeability to simple electrolytes, e.g. NaCl, some practically keeping back this salt, others allowing a very slow diffusion. These initial variations would be expected to influence more markedly the members of lower than of higher alcohol number. The accuracy attainable has not however been sufficiently tested in the case of these membranes of lower permeability.

By the use of this alcohol method, it is possible to prepare a series of membranes the members of which allow of slower and slower diffusion of a given substance. By continuing the series downwards a stage is reached where no diffusion at all of the particular substance takes place. Thus a given substance can be characterised by a number which represents the strength of alcohol required to produce a membrane which just prevents diffusion of the substance. Such a number may be termed the "alcohol index" for the particular substance. Some of these indices have been worked out, and are given in the following table:

Water	} —	Safranine	75–77·5
NaCl, NH_4Cl		Dextrin	85–87·5
$KMnO_4$	30–40	Starch	90
Picric acid	35–40	Aniline blue	92
Potassium oxalate ...	60–70	Litmus (neut.)	93
Bismarck brown ...	65 (−)	Congo red	96
Methylene blue ...	70 (−)	Night blue	>96
Neutral red	72·5–75		

Water, sodium chloride and ammonium chloride all pass through air-dried membranes. Passage of water is shown by endosmosis, also by the fact that water evaporates from a sealed air-dried membrane when the latter is hung up in the air. The rate of passage is small, and individual variations are large in proportion. Some of these membranes only allow the faintest trace of sodium chloride to pass through in a week; in the case of others passage can be detected in two days. It is doubtful if any of these membranes can be prepared so as to allow passage to water, and at the same time to hold back completely simple chlorides.

The strong endosmotic effect is very troublesome in working with such substances as potassium oxalate, especially in molar concentration. The internal liquid tends to overflow and the levels have to be continually readjusted. Most of the crystalloids tested seem to possess a diffusibility similar to that of potassium oxalate. Such were $CuSO_4$, Na_2SO_4, $K_4Fe(CN)_6$. A 60 % membrane allowed a slow diffusion of glucose and of barium chloride. This part of the investigation was not however carried out at all thoroughly.

It should be noted that membranes of index 50–60 % form very efficient semi-permeable membranes, i.e. while easily permeable to water, they hold back a large number of crystalloids. A membrane of this sort may be used to effect a dialytic separation of certain kinds of salts—thus sodium chloride can be readily dialysed away from a sodium chloride, sodium sulphate mixture. Whether such membranes would keep back these crystalloids under pressure remains to be seen.

It might be suggested that the relatively high impermeability of these membranes—say to molar sodium sulphate—is due to the effect of the strong salt solution upon the membrane, viz. to the withdrawal of water from the membrane by the osmotic action of the salt. This however is contradicted by the behaviour of strong sodium chloride solutions which should on this hypothesis have a similar dehydrating effect, by the magnitude of the endosmotic effect, and by the fact that a 60 % membrane after treatment with molar Na_2SO_4 is found to be still of number 60 % in its behaviour to potassium permanganate.

Only a very slow passage of starch is allowed by a 90 % membrane. Even a 92 % membrane to all practical purposes keeps back a starch solution during several days' dialysis. It is probable that many colloids are heterogeneous as regards degree of dispersion, so that a sharply defined "critical point" would not be expected.

The dialysates from 94 and 95 % membranes containing litmus show a peculiar feature. On comparing these with matching dilutions of the internal solution, they are found to give the same colour reaction to acids, but not to alkalis. Thus the addition of a drop of alkali to the matching solution produces a distinct blue; on similar treatment, the dialysates from 94 and 95 % membranes merely become slightly more violet. A similar behaviour, though not so marked, is seen in the dialysate from a 96 % membrane. It is suggested that litmus contains at least two fractions, one of which is more diffusible and gives a part only of the indicator reactions of the crude solution.

Equilibrium.

The question as to whether equilibrium between the collodion membrane and the alcohol solution has been reached in the 24 hours allowed for imbibition, has only been studied in the case of 72·5 % membranes. It was then found that no essential difference in permeability subsisted between membranes which had remained in contact with the alcohol solution for one day and membranes which had been similarly treated for 4 days. Thus in the case

of 72·5 % membranes, a state of equilibrium or zone of slow change has been reached in 24 hours. Higher in the series it is probable that an imbibition equilibrium is reached in a shorter time, but here the slow change due to solution of the membrane becomes more marked. In the case of membranes lower than 70 %, it should not be taken for granted that equilibrium in imbibition is reached after 24 hours' immersion in the alcohol solution.

Uses of these membranes.

Some of the more obvious uses to which these membranes may be put are

(1) replacement of the copper ferrocyanide membranes in osmometric work;

(2) dialysis of intermediate colloids, e.g. dextrin: more generally, the examination and separation of digestion products;

(3) carrying out a more exact study than has yet been made of the relations between the phenomena of diffusion and adsorption.

An experiment of the type suggested in (2) will serve to illustrate the potentialities of the method.

Six 92 % membranes were made and set up as follows:

(1) and (2) 0·1 % starch solution + equal bulk of water,
(3) and (4) 0·5 % dextrin solution + equal bulk of water,
(5) and (6) 0·5 % dextrin solution + 0·1 % starch solution in equal bulks.

These were tested each day over a period of four days, when it was found that in no case could starch be detected in the dialysates, whereas dextrin of strength 0·05 % dialysed through in each 24 hours in the case of membranes (3), (4), (5) and (6).

On the fifth day, each dialyser was washed and set up alongside a 50 % and an 85 % membrane, with a mixture in equal parts of the following:

0·5 % starch solution,
0·5 % dextrin solution,
0·2 m glucose solution,
1 m sodium chloride solution.

After 24 hours' dialysis, the following scheme represents the condition of the dialysates:

Test	50 %	85 %	92 %	Internal
$AgNO_3$	fairly strong precipitate	strong precipitate	strong precipitate	strong precipitate
Fehling's solution	neg.	strong reduction	—	—
I_2 in KI	coloration = water control	coloration = water control	strong red coloration	intense blue

The application of these membranes to more difficult problems of analysis may be anticipated.

All the work hitherto described was carried out with an 8 % collodion solution in equal parts of ether and alcohol. Other collodion solutions have been tested so far as to indicate what possibilities they present.

Acetone collodion; 8 % solution; dispersion takes place with rapidity, giving a solution which is more mobile than the corresponding ether-alcohol solution.

The membranes prepared from this solution in the ordinary way are white throughout, show normal amount of irregularity in thickness in different parts, have a small water percentage and comparatively low permeability in wet condition and show small shrinkage (6 %) on drying. In the wet condition the thickness is 8–10 μ, and after drying 5–7 μ.

When treated by the alcohol method, a more gradual variation with alcohol percentage is obtained, the interval 0 %—70 % in the case of ether-alcohol membranes corresponding with the interval 0 %—90 % with acetone membranes.

These membranes appear to be of practical utility, especially in respect of their more gradual grading below 90 %.

Ether collodion; 8 % solution; dispersion does not occur so rapidly as in acetone; the resultant solution is very viscous, more so than the ether-alcohol solution. The membranes were prepared in the usual way; they are white, give extremely small shrinkage on drying, and when freshly prepared are permeable only to water and a few salts. They are extremely difficult to prepare on account of the high impermeability which prevents the rapid access of water to the underlying layers. By the action of water the collodion film becomes hard and can with difficulty be removed from the tube.

On treatment with alcohol, these membranes show a behaviour similar to that of the acetone-collodion membranes described above, i.e. they respond more sluggishly to the alcohol treatment.

This last feature is considered to be of interest in respect of the general technique adopted. It would explain the tendency observed in certain membranes to be more permeable towards the closed end, where, on account of the conditions obtaining during their manufacture, they tend to contain a lower percentage of ether at the time of fixation in water.

Alcohol collodion. The 8 % solution was so viscous that a 4 % solution was employed. The latter is rather more fluid than the normal 8 % ether-alcohol solution.

The membranes were held for a few seconds in air, and then fixed in

water. They are glass-clear and in the wet condition are highly permeable, e.g. to starch. In spite of the thin solution employed, wet membranes are extremely thick (200–300 μ), and have a very high water-content (dry weight = 100, wet weight = 1400). The shrinkage on drying is enormous (60–70 %).

The interest of these membranes is that they form the end term of the series; ether membrane → membrane of type (5, 0) → membrane of type (5, 1) → alcohol membrane.

Gradation by means of acetone-water mixtures.

No practicable results can be got by using acetone-water in place of alcohol-water mixtures. In 60 % acetone (and higher concentrations) membranes of any type become unmanageably soft (the clear normal type of membrane becoming white in this process); in 40 % acetone, the membranes soften, shrink to very small dimensions, and on transferring to water become hard and highly impermeable.

Membranes made inside the tube.

These can be prepared conveniently as follows. Two tubes of the same diameter are passed through a cork as in Fig. 1. One of these is filled with 8 % collodion solution. On turning upside down and allowing to drain, a film is left inside the upper tube. Fifteen minutes drainage was allowed. The tube with the film is then taken out, and air blown in by means of a pipette until the whole film is nearly dry (one minute is about sufficient); the free end of the membrane is now disengaged with a knife and a little further blowing causes the whole membrane to come away. These membranes are simply dried out in air without coming in contact with water. They are of the clear alcohol type, but do not show shrinkage as they had already almost dried while in contact with the glass, which restrains shrinkage in a mechanical way.

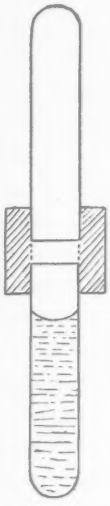

Fig. 1

These membranes can with skill be prepared of great uniformity (apart from the usual tendency to be thinner at the closed than at the open end), they are very thin, they can be differentiated in alcohol in the usual way, and their use is recommended in cases where a rapid dialysis is imperative and where adsorption of a particular substance on the membrane is required to be

reduced as much as possible. These membranes, on account of their extreme thinness, are rather fragile.

Thin sheet collodion can be readily prepared in this way, viz. by forming a membrane inside a large cylindrical vessel and treating in the above manner. After drying, the film is simply cut down one side with a knife and taken out.

Method of grading membranes by controlling the amount of drying.

This method was the one first adopted. It bears certain resemblances to Schoep's method. In fact, in the early stages, glycerol solutions were used, the wet membranes as prepared in the first instance being soaked in glycerol solutions of different strengths and then hung up in air to dry. The higher the percentage of glycerol the greater the permeability of the resultant membrane.

This method was later superseded by the following. A solution of $CaCl_2$, as nearly as possible saturated and with alkalinity neutralised, was prepared, filtered, and placed in the bottom of a large desiccator. This solution may be designated *A*. The wet membranes were soaked for 24 hours in various dilutions of *A*, and then hung up over the concentrated solution in the desiccator; the latter is then exhausted. By this means the attainment of equilibrium is expedited. It is obvious that a membrane which has imbibed a 5 % dilution of *A* loses more water in reaching equilibrium with *A* than a membrane which has been soaked in a 10 % dilution, and would accordingly be expected to be less permeable. The membranes are finally washed and are then ready for use.

The practical features of the process are as follows:

The $CaCl_2$ solution should be as strong as possible: with weak solutions the attainment of equilibrium is slower. Before the membranes are hung up to dry in the desiccator, all excess drops of the diluted $CaCl_2$ solution must be wiped off. Only in this way can uniformity be obtained.

As regards results, this method is distinctly good. It is necessary however that the membranes before treatment be of uniform permeability throughout. Any marked variations will be reproduced in the graded membranes. In fact in the present work the variations in different parts of the freshly prepared membranes as described on p. 596 were first discovered by the variations which appeared in different parts of the membranes, the permeability of which had been reduced in this way. In order to secure uniform membranes to begin with, the freshly prepared membranes were soaked in a 94 % alcohol solution.

A membrane which has been soaked in 94 % alcohol, then graded down by using a 7·5 % *A* solution just holds back methylene blue, i.e. it corresponds to a membrane treated with 70 % alcohol.

Apart from its laboriousness and its limited applicability, this method may be objected to inasmuch as the removal of the calcium chloride from the graded membrane may be slow, and may not take place at all in some cases. This objection could be met by using strong solutions of sodium chloride instead. There is a possibility that such a method may be of use in preparing membranes for osmometric work.

Summary.

Collodion thimbles of regularly increasing degree of permeability may be prepared by soaking air-dried thimbles in alcohol-water mixtures of increasing alcohol content.

The diffusive capacity of any substance through collodion may be specified in terms of the alcohol strength required to produce the membrane which just prevents its passage. This may be termed the "alcohol index" of the substance.

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The University of Chicago Press

The Cambridge University Press
Agents for the British Empire
London, Fetter Lane

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